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13. ABSTRACT ***(Maximum 200 Words)***

Crucial steps in tumor progression and the process of metastasis, e.g. tumor growth, invasion through extracellular matrices and angiogenesis, involve proteolytic modification of the pericellular matrix surrounding tumor cells. A major class of proteases involved in these processes is the matrix metalloproteinases (MMPs), and inhibition of MMPs prevents progression and metastasis of several tumor types, including human breast carcinomas, in animal models. In vivo, tumor MMPs are usually produced by stromal cells associated with tumors rather than the tumor cells themselves. The tumor cell surface glycoprotein, EMMPRIN, stimulates MMP production by fibroblasts and endothelial cells, and may be an important regulator of MMP production during tumorigenesis in vivo. However no direct evidence for its role in tumor progression had been published prior to this study. The focus of this proposal has been to demonstrate directly whether or not EMMPRIN promotes breast cancer progression, whether a role for EMMPRIN in tumor progression may be to promote or induce angiogenesis, and whether approaches can be developed that may have future therapeutic potential. This study has shown that EMMPRIN promotes tumor growth and invasion in an animal model and that interference with the action of EMMPRIN may be an effective way to retard breast carcinoma progression in patients.

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INTRODUCTION

Crucial steps in tumor progression and the process of metastasis, e.g. tumor growth, invasion through extracellular matrices and angiogenesis, involve proteolytic modification of the pericellular matrix surrounding tumor cells. A major class of proteases involved in these processes is the matrix metalloproteinases (MMPs), and inhibition of MMPs prevents progression and metastasis of several tumor types, including human breast carcinomas, in animal models. In vivo, tumor MMPs are usually produced by stromal cells associated with tumors rather than the tumor cells themselves. The tumor cell surface glycoprotein, EMMPRIN, stimulates MMP production by fibroblasts and endothelial cells, and may be an important regulator of MMP production during tumorigenesis in vivo. However no direct evidence for an important role in tumor progression has been published. The focus of this proposal will be to demonstrate directly whether or not EMMPRIN promotes breast cancer progression and whether a role for EMMPRIN in tumor progression may be to promote or induce angiogenesis. This study should determine definitively whether EMMPRIN-mediated regulation of MMPs may constitute a newly discovered step in breast carcinoma progression and metastasis. Interference with EMMPRIN action may then be an effective way to retard breast carcinoma progression in patients.

Abbreviations used: EMMPRIN, extracellular matrix metalloproteinase inducer; MMP, matrix metalloproteinase; MMP-1, interstitial collagenase; MMP-2, gelatinase A

BODY

Original Statement of Work Task 1: To document that increased expression of EMMPRIN in non-aggressive human breast carcinoma cells leads to enhanced tumor progression.

As proposed in our original Statement of Work, we have tested whether tumor growth and metastasis are affected when EMMPRIN expression is increased in non-aggressive MDA-MB436 human breast carcinoma cells. In previous studies we demonstrated that EMMPRIN stimulates production of MMPs in fibroblasts, possibly explaining the finding that most MMPs are produced by tumor stromal fibroblasts rather than by tumor cells themselves. We have now shown that EMMPRIN promotes tumor progression in vivo. Human MDA-MB-436 breast cancer cells, which are slow growing in vivo, were transfected with EMMPRIN cDNA and injected orthotopically into mammary tissue of female nu/nu mice. Green fluorescent protein (GFP) was used to visualize metastases. Breast cancer cell clones transfected with EMMPRIN/GFP cDNA gave rise to much larger tumors than GFP- or plasmid-transfected cancer cells, although the EMMPRIN transfectants grew at the same rate as controls under routine culture conditions. The EMMPRIN transfectants were more invasive both in vivo and in in vitro invasion assays, and gave rise to metastases in approximately 25% of the experimental animals. Control animals showed no signs of invasiveness or metastases. Mortality was 100% in experimental and insignificant in controls over a 15-week period. Increased MMP expression was also demonstrated in EMMPRIN-enhanced tumors. Details of these experiments are given in a published manuscript, attached in Appendix 1.

In a collaborative study, we also examined the relationship of EMMPRIN and MMPs to

human brain glioma progression, especially with respect to differential expression of EMMPRIN in angiogenic and blood-brain barrier endothelial cells (details in published manuscript, Appendix 2). In addition, in this study we showed for the first time that EMMPRIN stimulates membrane-type MMPs that have recently been shown to play important roles in activation of MMP-2 and in tumor invasiveness (details in published manuscript, Appendix 3). In more recent work, we have shown that this stimulation also takes place in human breast cancer cells, and leads to increased invasiveness and anchorage-independent growth of these cells in an autocrine manner (reported in DAMD17-99-1-9411, trainee grant for Erica Marieb).

Further extension of this work, as outlined in the original Statement of Work is being performed with new funding from the National Cancer Institute (CA79866). A new Statement of Work was approved as of March 23, 2000.

Original Statement of Work Task 2: To document whether inhibition of EMMPRIN expression in malignant human and murine mammary carcinoma cells blocks tumor growth and/or metastasis in vivo.

As proposed, antisense cDNA and ribozyme constructs were produced for mouse and human EMMPRIN, as well as sense controls. Stable transfectants of murine TA3/St mammary carcinoma cells (an aggressive cancer cell line) were produced using these constructs. Unfortunately, complete inhibition of EMMPRIN expression has not yet been obtained. Clones were isolated that exhibited partially inhibited expression and these were used to determine whether metastasis to the lung after intravenous injection into the tail of syngeneic mice was affected. These experiments have not been effective. Continuation of this work is being performed with new funding from the National Cancer Institute (CA79866). A new Statement of Work was approved as of March 23, 2000.

Original Statement of Work Task 3: To test whether EMMPRIN stimulates endothelial morphogenesis in a 3-dimensional collagenous matrix in culture.

We have set up the methods and have performed initial experiments that support our proposal. In these experiments, human umbilical vein endothelial cells were cultured on type I collagen gels, then treated with either bFGF, a known angiogenic agent, or purified EMMPRIN. This method is a standard technique that mimics aspects of angiogenesis, i.e. invasion of a three-dimensional matrix and formation of capillary-like tubules. In our experiments we have observed that 1 μg of EMMPRIN duplicates the effect of 5 ng of bFGF in its ability to initiate capillary-like tubule formation. We believe that the relatively high amount of EMMPRIN required is due to inactivation of most of the protein during purification.

In the course of producing EMMPRIN preparations, and investigating potential binding partners, we discovered that EMMPRIN avidly binds collagenase (MMP-1). In this study we have found that EMMPRIN not only stimulates production of interstitial collagenase (MMP-1) but also forms a complex with MMP-1 at the tumor cell surface. Complex formation was demonstrated by phage display, affinity chromatography, and immunocytochemistry. Presentation of MMP-1 complexed to EMMPRIN at the tumor cell surface may be important in modifying the tumor cell pericellular matrix to promote invasion. Details of these experiments

are given in a published manuscript, attached in Appendix 4.

Since EMMPRIN preparations are onerous to make and subject to denaturation, and since some EMMPRIN preparations have now been shown to contain MMP-1 (see above and Appendix 4), we have switched to a new technique for studying this phenomenon. We have found that infection of fibroblasts with recombinant EMMPRIN adenovirus is a very effective means to stimulate MMP production, due to mutual interaction between neighboring cells expressing both EMMPRIN and EMMPRIN receptor. We are now using this approach with human endothelial cells. That is, we infect endothelial cells with the adenovirus, then monitor expression of EMMPRIN and MMPs. We will determine the effect of the adenoviral-delivered EMMPRIN on capillary tubule formation. This approach is now supported by new funds from the National Cancer Institute (CA79866) (Revised Statement of Work).

Revised Statement of Work Task 1: To test the effects of a peptide antagonist of EMMPRIN action on tumor growth and invasion in animal tumor models.

We have attempted to map the active site of the EMMPRIN molecule by testing the effect on MMP production of synthetic peptides with sequences from within the extracellular domain of the EMMPRIN molecule. The aim of this approach was to determine whether synthetic peptides from the active site would antagonize or mimic the action of EMMPRIN. Previous results suggested that we had obtained an inhibitory peptide. However it turns out that the activity of the peptide depends on the way in which it is tested; it is inhibitory in cocultures of tumor cells and fibroblasts but stimulatory when applied directly to fibroblasts alone. We are currently attempting to understand this unexpected finding.

Despite the confusing results above, the active peptides have been useful for ligand chromatographic isolation of an EMMPRIN-binding protein. This protein has been identified as annexin II, and we are currently attempting to determine the role of EMMPRIN-annexin II interaction in EMMPRIN action.

Revised Statement of Work Task 2: To explore the use of recombinant adenoviral constructs for efficient delivery.

We have produced recombinant adenoviruses driving expression of EMMPRIN and mutated forms of EMMPRIN to further map the active site of EMMPRIN. In addition these adenoviruses can be used for delivery in tests of EMMPRIN function. In this way we have found that an adenoviral construct driving expression of a soluble form of EMMPRIN (lacking transmembrane and cytoplasmic domains) lacks EMMPRIN activity and may act as a dominant negative inhibitor. We are currently testing its ability to block EMMPRIN function in vitro and in vivo.

Production of recombinant EMMPRIN adenoviruses has also allowed us to study EMMPRIN function further. This has been done with human EMMPRIN and murine EMMPRIN (basigin). Studies of the effects of recombinant human EMMPRIN adenovirus have been reported under DAMD17-99-1-9411 (trainee grant for Erica Marieb). Analysis of basigin-null mice has shown that it is involved in several important physiological processes including reproductive, immune and neural activities. However, its molecular mechanism of action in these

processes has not yet been established. Our objective here was to determine whether basigin has functional properties similar to its apparent human tumor cell homolog, EMMPRIN, i.e. the ability to stimulate MMP production in fibroblasts. We found that mouse cells express two major forms of basigin that differ in their degree of glycosylation (molecular weights: 45 kD and 58 kD) but, in similar fashion to human EMMPRIN, mouse tumor cells express higher levels of basigin than normal cells. We have used three different methods to show that basigin stimulates MMP expression in fibroblasts. First, recombinant basigin was partially purified from transfected CHO cells by affinity chromatography. This basigin preparation stimulates production of MMPs on addition to fibroblasts in culture. Second, co-culture of basigin-transfected CHO cells with fibroblasts gives rise to increased expression of MMPs as compared to control co-cultures. Third, we employed a novel approach in which a recombinant basigin adenovirus was constructed and used to infect the target fibroblasts, so that mutual stimulation between neighboring fibroblasts would be expected to result. In this method also, basigin stimulates production of MMPs. Finally, we showed that addition of anti-basigin or anti-human EMMPRIN antibody, respectively, to recombinant basigin or human EMMPRIN adenovirus-infected cells augments stimulation of MMP synthesis, implying that cross-linking of basigin/EMMPRIN in the membrane enhances activity. We conclude that murine basigin and human EMMPRIN have similar MMP-inducing activities and are functional homologs. Details of these experiments are given in a published manuscript, Appendix 5).

KEY RESEARCH ACCOMPLISHMENTS

1) Demonstration that increased expression of EMMPRIN leads to increased tumor growth and invasion in vivo
2) Demonstration that EMMPRIN stimulates membrane-type MMP production and activation of MMP-2
3) Demonstration that EMMPRIN is differentially expressed by human angiogenic and blood-brain barrier endothelial cells.
4) Demonstration of specific binding of EMMPRIN to collagenase (MMP-1)
5) Demonstration of specific binding of EMMPRIN to annexin II
6) Development of an efficient recombinant adenovirus-based system for testing the cellular effects of EMMPRIN and EMMPRIN mutants
7) Demonstration that murine and human homologs of EMMPRIN have similar MMP-inducing properties.

REPORTABLE OUTCOMES

1) Peer-Reviewed Publications:

Guo, H., Li, R., Zucker, S., and Toole, B.P.: EMMPRIN (CD147), an inducer of matrix metalloproteinase synthesis, also binds interstitial collagenase (MMP-1) to the tumor cell surface. Cancer Res. **60**: 888-891, 2000.

Sameshima, T., Nabeshima, K., Toole, B.P., Yokogami, K., Okada, Y., Goya, T., Koono, M., and Wakisaka, S.: Expression of EMMPRIN (CD147), a cell surface inducer of matrix metalloproteinases, in normal human brain and gliomas. Int. J. Cancer **88**:21-27, 2000.

Sameshima, T., Nabeshima, K., Toole, B.P., Yokogami, K., Okada, Y., Goya, T., Koono, M., and Wakisaka, S.: Gliomal cell extracellular matrix metalloproteinase inducer (EMMPRIN) (CD147) stimulates production of membrane-type matrix metalloproteinases and activation of gelatinase A in co-cultures with brain-derived fibroblasts. Cancer Lett. **157**: 177-184, 2000.

Li., R., Huang, L., Guo, H., and Toole, B.P.: Basigin (murine EMMPRIN) stimulates matrix metalloproteinase production by fibroblasts. J. Cell. Physiol.**186**: 371-379, 2001.

Zucker, S., Hymowitz, M., Rollo, E.E., Mann, R., Conner, C.E., Cao, J., Foda, H.D., Tompkins, D.C., and Toole, B.P.: Tumorigenic potential of extracellular matrix metalloproteinase inducer (EMMPRIN). Amer. J. Pathol., **158**: 1921-1928, 2001.

Invited Reviews:

Toole, B.P. and Zucker, S.: EMMPRIN, a tumor cell surface inducer of matrix metalloproteinase production in stromal cells. Cancer Research Alert, **2**: 13-17, 2000.

Toole, B.P.: Emmprin, an inducer of matrix metalloproteinase synthesis, promotes tumor progression. In: "Cell Invasion", ed. J. Heino & V.M. Kahari, Landes Bioscience, in press.

Toole, B.P.: Emmprin, a tumor cell surface regulator of matrix metalloproteinase production, In: "Cell Surface Proteases", ed. S. Zucker and W.T. Chen, Academic Press, in press.

2) Presentations:

FASEB conference symposium on Misregulation of the Basement Membrane in Human Disease, April 2000: "Regulation of tumor progression by EMMPRIN, a tumor cell surface inducer of matrix metalloproteinases" by Bryan Toole

Plenary Lecture to the Finnish Cancer Society, October, 2000: "Emmprin and hyaluronan-tumor cell interactions: new targets for therapeutic intervention?" by Bryan Toole

Symposium on Membrane Proteases and Cancer, Sicily, May, 2001: "Emmprin, a tumor cell surface inducer of MMP production, promotes tumor progression" by Bryan Toole

3) New funding:

National Cancer Institute (CA 79866): "Tumor cell-stromal interactions in cancer"

CONCLUSIONS

We conclude from the above work that EMMPRIN promotes mammary carcinoma progression via stimulation of MMP production in tumor stromal cells. Our data suggest that one mechanism whereby EMMPRIN may act is by presentation of interstitial collagenase on the tumor cell surface. Our recent data suggest that EMMPRIN-derived peptides or soluble forms of EMMPRIN may be efficacious as inhibitors of EMMPRIN action and therefore may be useful therapeutically.

REFERENCES: None

PERSONNEL:
The personnel receiving salary from this grant were:
Bryan P. Toole, Ph.D.
Huiming Guo, Ph.D.

APPENDICES:

1. Zucker, S., Hymowitz, M., Rollo, E.E., Mann, R., Conner, C.E., Cao, J., Foda, H.D., Tompkins, D.C., and Toole, B.P.: Tumorigenic potential of extracellular matrix metalloproteinase inducer (EMMPRIN). Amer. J. Pathol., **158**: 1921-1928, 2001.

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APPENDIX 1:

Zucker, S., Hymowitz, M., Rollo, E.E., Mann, R., Conner, C.E., Cao, J., Foda, H.D., Tompkins, D.C., and Toole, B.P.: Tumorigenic potential of extracellular matrix metalloproteinase inducer (EMMPRIN). Amer. J. Pathol., **158**: 1921-1928, 2001.

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Short Communication

Tumorigenic Potential of Extracellular Matrix Metalloproteinase Inducer

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From the Departments of Research and Medicine, Veterans Affairs Medical Center, Northport, New York; Department of Medicine,† State University of New York at Stony Brook, Stony Brook, New York; and the Department of Anatomy and Cellular Biology,‡ Tufts University School of Medicine, Boston, Massachusetts*

Extracellular matrix metalloproteinase inducer (EMMPRIN), a glycoprotein present on the cancer cell plasma membrane, enhances fibroblast synthesis of matrix metalloproteinases (MMPs). The demonstration that peritumoral fibroblasts synthesize most of the MMPs in human tumors rather than the cancer cells themselves has ignited interest in the role of EMMPRIN in tumor dissemination. In this report we have demonstrated a role for EMMPRIN in cancer progression. Human MDA-MB-436 breast cancer cells, which are tumorigenic but slow growing *in vivo*, were transfected with EMMPRIN cDNA and injected orthotopically into mammary tissue of female NCr *nu/nu* mice. Green fluorescent protein was used to visualize metastases. In three experiments, breast cancer cell clones transfected with EMMPRIN cDNA were considerably more tumorigenic and invasive than plasmid-transfected cancer cells. Increased gelatinase A and gelatinase B expression (demonstrated by *in situ* hybridization and gelatin substrate zymography) was demonstrated in EMMPRIN-enhanced tumors. In contrast to *de novo* breast cancers in humans, human tumors transplanted into mice elicited minimal stromal or inflammatory cell reactions. Based on these experimental studies and our previous demonstration that EMMPRIN is prominently displayed in human cancer tissue, we propose that EMMPRIN plays an important role in cancer progression by increasing synthesis of MMPs. *(Am J Pathol 2001, 158:1921–1928)*

Extracellular matrix metalloproteinase inducer (EMMPRIN) was originally designated tumor collagenase stimulating factor (TCSF) by Biswas et al[1] after isolation and purification of the 58-kd glycoprotein from the plasma membrane of cancer cells and demonstration of its function in stimulating fibroblast synthesis of collagenase-1 (MMP-1). The subsequent finding that EMMPRIN also induced fibroblast synthesis of gelatinase A (MMP-2) and stromelysin-1 (MMP-3) indicated a more general effect on the production of MMPs.[2] Recent studies have documented the capacity of recombinant EMMPRIN or EMMPRIN purified from cancer cells to stimulate fibroblast production/secretion of stromelysin-1, collagenase-1, and gelatinase A *in vitro*.[2,3] After secretion from fibroblasts, collagenase-1 is able to bind to EMMPRIN on the tumor cell surface.[4] The demonstration by *in situ* hybridization (mRNA localization) that peritumoral fibroblasts synthesize most of the MMPs (collagenases, gelatinases, stromelysins, and membrane type-MMPs) in human tumors rather than the cancer cells themselves has ignited interest in the role of EMMPRIN in tumor dissemination.[5,6] The association of intense EMMPRIN expression in neoplastic cells within invasive human tumors[7,8] further supports a role for EMMPRIN in cancer dissemination. These data are consistent with a central function for EMMPRIN in stimulating stromal cell production of MMPs which, after pericellular activation, directly degrade the extracellular matrix.[1]

Peptide sequencing and cDNA isolation of EMMPRIN from tumor cells[1,9] led to the recognition that EMMPRIN is identical to human basigin[10] and M6 antigen,[11] proteins of previously unknown function that were identified by other investigators in embryonic and inflammatory tissues. A knockout mouse has been produced in which the murine homologue of basigin/EMMPRIN is lacking.[12] The null mutant is, in most cases, unable to undergo oocyte implantation, presumably due to the requirement for MMPs in this process. It is apparent that although many embryonic and adult tissues express EMMPRIN, the level of EMMPRIN expression and glycosylation in tumors is

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much greater than in corresponding normal tissues.[7,13–15]

In the current study we have examined the function of EMMPRIN in a cancer model in immunodeficient mice. Human MDA-MB-436 breast cancer cells that are tumorigenic, estrogen independent, and moderately invasive *in vitro*, but slow growing *in vivo*,[16] were transfected with EMMPRIN cDNA and injected orthotopically into the mammary fat pad of nude mice. We took advantage of the observation that the 29-kd green fluorescent protein (GFP) of the jellyfish *Aequoria victora* retains its fluorescent properties when recombinantly expressed in eukaryotic cells[17] along with EMMPRIN cDNA and can be used as a powerful marker for gene expression and cancer dissemination *in vivo*. Cancer cells transfected with both EMMPRIN cDNA and GFP cDNA were compared with cancer cells transfected with GFP cDNA alone for tumorigenic behavior. The results demonstrated that tumor growth in nude mice was considerably enhanced by EMMPRIN/GFP- transfected breast cancer cells as compared to cells transfected with GFP alone.

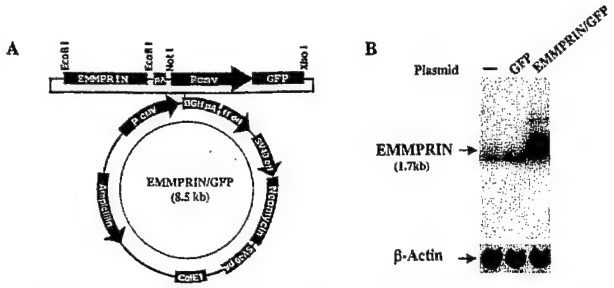


Figure 1. A: Schematic illustration of the EMMPRIN/GFP plasmid. A 1.6-kb cDNA representing the entire EMMPRIN sequence was placed at an *Eco*R 1 site under the control of the CMV promoter in pcDNA 3. GFP cDNA was inserted along with an upstream CMV promoter into the EMMPRIN expression vector between *Not*I and *Xho*I sites. A polyadenylation (PA) signal was placed downstream. **B:** Northern blot analysis of EMMPRIN. Approximately 20 μg of total cellular RNA from plasmid alone-transfected, GFP-transfected, and EMMPRIN/GFP-transfected MDA-MB-436 breast cancer cells (from experiment 3) was size fractionated in a 1% denaturing agarose gel, transferred to a nylon membrane, and incubated with 1.7 kb of ^{32}P-radiolabeled EMMPRIN cDNA as a probe. Blots were analyzed by autoradiography. A single 1.7-kb mRNA transcript corresponding to the known EMMPRIN band was detected at ~20× greater intensity in EMMPRIN/GFP-transfected cells as compared to plasmid alone or GFP-transfected cells.

Materials and Methods

Reagents

Restriction enzymes were purchased from Stratagene (La Jolla, CA). EMMPRIN was purified from LX-1 lung cancer cells using affinity column chromatography.[18] Monoclonal antibodies to EMMPRIN (clone 1G6.2) were produced in collaboration with Dr. D. Dembro at Chemicon International, Inc. (Temecula, CA). The F4/80 rat anti-mouse macrophage antibody was purchased from Serotec (Raleigh, NC). Thrombin was a kind gift from Dr. J. Jesty. Phorbol 12- myristate-13 acetate (PMA) was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines and Culture Conditions

Human MDA-MB-436 breast cancer cells were maintained in Richter's improved minimal essential medium supplemented with 10% donor calf serum.[16] Immunostaining of MDA-MB-436 cells was performed using a primary mouse monoclonal antibody to EMMPRIN (1G6.2) and a secondary goat anti-mouse IgG (H&L) horseradish peroxidase-labeled antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Construction of Plasmids and Transfection into Cells

A 1.6-kb cDNA[1] representing the entire EMMPRIN sequence encoding 269 amino acid residues was placed at an *Eco*RI site under the control of the CMV promoter in pcDNA3 (Invitrogen, Carlsbad, CA). To facilitate identification of transfected cells *in vitro* and metastases *in vivo*, GFP (GFPmut1 variant) cDNA (Clontec Laboratory, Inc., Palo Alto, CA) was inserted into the EMMPRIN-containing plasmid. The GFP cDNA, along with a separate upstream cytomegalovirus promoter from pEGFP-C1 plasmid (Clontec), was inserted into the EMMPRIN expression vector between *Not*I and *Xho*I sites as shown in Figure 1A. An additional polyadenylation signal from pSG5 (Stratagene) was placed downstream of the *EMMPRIN* gene to provide balanced expression of both recombinant genes under control of CMV promoters. The resulting plasmid was named EMMPRIN/GFP. As a control plasmid, GFP cDNA alone was subcloned into pcDNA3 without EMMPRIN cDNA. In experiment 2, EMMPRIN cDNA was subcloned into pcDNA3 without GFP; the control plasmid was pcDNA3 alone.

The human MDA-MB-436 breast cancer cell line was stably transfected using the calcium phosphate precipitation method.[19] Selected G418-resistant clones were screened by fluorescent appearance using a Nikon microscope equipped with a xenon lamp power supply and a GFP filter set. Fluorescent positive clones were further analyzed by Northern blot analysis probed with an EMMPRIN cDNA fragment.

RNA Isolation and Northern Blot Hybridization

Total RNA was extracted from MDA-MB-436 cells stably transfected with desired plasmids by guanidine solubilization, phenol/chloroform extraction, and serial precipitation.[1,20] Approximately 20 μg of total RNA was resolved by denaturing gel electrophoresis followed by Northern transfer to nylon membranes (Schleicher and Schuell, Keene, NH). Blots were hybridized to ^{32}P-radiolabeled EMMPRIN cDNA (1.7 kb) at 42°C as described[20] and analyzed after overnight exposure with an intensity screen at −80°C. The amount of the samples applied to the lanes was normalized by β-actin RNA.

Labeling of RNA Probes

Antisense and sense digoxigenin-labeled RNA probes for EMMPRIN, gelatinase A, and gelatinase B were syn-

thesized by reverse-transcribing 1 μg of cDNA from a polymerase chain reaction that had used gene-specific primers that contain the T7 or T3 phage promoter sequence followed by 20–25 bases of the mRNA sequence.[21] The probes for human EMMPRIN (bases 319–701), human gelatinase A (bases 42–436), and mouse gelatinase B (bases 56–361) were designed based on published nucleotide sequences (GenBank accession numbers AH007299, J03210, and Z27231, respectively). Homology between the human and mouse nucleotide sequences for gelatinase A and gelatinase B are 91 and 78%, respectively, as determined by BLAST 2 sequence alignment (www.ncbi.nlm.nih.gov/gorf/bl2.html). *In vitro* transcription of the amplified DNA template was performed using the digoxigenin RNA labeling kit (Roche Molecular Biochemicals, Indianapolis, IN). Labeled probes were purified and sequences were verified.

In Situ *Hybridization*

Serial sections of paraffin-embedded mouse tumors were prepared for *in situ* hybridization according to the method of Komminoth.[22] Slides were processed for immunodetection using anti-digoxigenin alkaline phosphatase conjugate antibody (Roche Molecular Biochemicals) and then incubated with substrate solution (Wash and Block Set; Roche Molecular Biochemicals).

Cell Proliferation in Vitro

Cell proliferation assays were performed by plating MDA-MB-436 cells at 4×10^4 cells per well (Costar, Corning, NY) and then switched to serum-free medium. After 48 hours, serum-enriched medium was added again and cells were cultivated for 4 additional days. Cell counts were performed daily.

Tumor Formation in Mice and Preparation of Tissue Extracts

Four-week-old female athymic NCr *nu/nu* mice were obtained from Taconic Farms (Germantown, NY). Cancer cells (1×10^6) were injected into the mammary fat pad of nude mice. Tumor growth was monitored weekly. Tumor volume was calculated using the formula: (length) (width^2)/2. At termination of experiments, mice were sacrificed, and autopsied; and tissue sections of the primary tumor, lungs, liver, lymph nodes, gastrointestinal tract, and other suspicious areas were prepared for histological/microscopic examination (hematoxylin and eosin staining of paraffin-embedded sections). Tissue sections were also stored in liquid nitrogen for subsequent *in situ* hybridization (see above) and extraction of MMPs. The extraction procedure for tumor tissue involved detergent and heat-extraction steps.[23]

Zymography and Immunohistochemistry

Primary cell cultures were transferred to serum-free medium and cultivated for 18 hours with or without the addition of thrombin or PMA. Serum-free spent medium was then collected and tested by gelatin zymography. Gelatin substrate zymography was preformed in 10% polyacrylamide gels that had been cast in the presence of 0.1% gelatin (NOVEX, San Diego, CA).[24,25] Protein determinations were made using the bicinchoninic acid reagent (Pierce, Rockford, IL).

Immunohistochemistry for mouse macrophages, monocytes, and dendritic cells was performed using the rat anti-mouse F4/80 antibody (Serotec) as described by Tsuruga et al.[26] A biotinylated rabbit anti-rat IgG was used as the secondary antibody. Immunoreactivity was visualized by the avidin-biotin peroxidase complex method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA).

Analysis of variance and Student's *t*-test were used to compare differences between groups in various experiments; $P < 0.05$ was considered significant. Survival experiences between groups were compared by the Wilcoxen χ^2 test.

Results

Cell Transfection and Proliferation

Northern blot analysis using EMMPRIN cDNA as a probe detected ~20-fold enhanced EMMPRIN expression by EMMPRIN/GFP-transfected cells as compared to GFP or non-transfected cells (Figure 1B). Similar results were achieved with each of the three transfected clones used in experiments 1 to 3 (see below). Immunostaining of MDA-MB-436 cells using specific mouse monoclonal antibodies to EMMPRIN documented intense staining of EMMPRIN/GFP-transfected cells and infrequent weak staining of GFP-transfected or vector transfected cells (data not shown).

There were no significant differences in cell doubling times between GFP and EMMPRIN/GFP cDNA-transfected cells (~18 hours) in medium with or without serum. These data are inconsistent with EMMPRIN acting as an autocrine growth factor for tumor cells *in vitro*.

Tumor Growth in Nude Mice

Three independent experiments, each using a different clone of EMMPRIN-transfected MDA-MB-436 cells, were performed. In experiments 1 and 2, the GFP-alone or vector-transfected clones did not form palpable tumors by the time of the experiment's termination at 12 weeks; however, ~0.01-cm^3 noninvasive tumors were identified at autopsy in 18 of 18 mice. In contrast, the EMMPRIN/GFP- or EMMPRIN (alone)- transfected clones formed palpable breast tumors at the site of mammary injection by week 6 in 18 of 18 mice which grew progressively to >1.7 cm^3 in diameter by week 12, at which time the animals were sacrificed. Histological examination of tissue sections revealed local cancer invasion, but no metastases.

In experiment 3, groups of 10 mice were injected with transfected MDA-MB-436 cells into mammary tissue. The

tumors emanating from the EMMPRIN/GFP cDNA-transfected MDA-MB-436 cells grew relatively rapidly, and all mice expired or had to be sacrificed within 12 weeks (Figure 2A). Extensive metastases to the liver, mediastinum, pleura, spleen, lymph nodes, and mesentery were present in 3 of 10 mice. In contrast, injection of the GFP cDNA-transfected tumor cells into mice resulted in tumors that grew considerably more slowly than EMMPRIN/GFP expressing tumors. Tumor diameter was <0.3 cm^3 and no metastases were noted at week 15 in 9 of 10 GFP-transfectant mice. One mouse in the GFP-transfected group developed a 1.4 cm^3 primary tumor by week 12. EMMPRIN/GFP and GFP expressing tumors (primary tumors and metastases) were readily visible by their expression of green fluorescence when examined grossly with fluorescent light (Figure 2B). The enhancement effect of GFP on tumor visualization has been previously described.[27]

Gelatinolytic Activity Extracted from Tumor Tissues and Cells

Gelatin zymograms of conditioned medium from 18-hour cultivated MDA-MB-436 tumor cells (Figure 2C, left panel) revealed that cells transfected with EMMPRIN/GFP cDNA secreted more than threefold more progelatinase A (72 kd) than did GFP cDNA-transfected cells. Treatment of both sets of transfected cells with thrombin (20 nmol/L) enhanced both secretion and activation of progelatinase A (more prominently displayed in the EMMPRIN/GFP-transfected cells). Treatment of cells with PMA (100 nmol/L) resulted in the appearance of weak gelatinolytic bands at 92 kd consistent with human progelatinase B.

Extracts of tumors derived from EMMPRIN/GFP cancer cell injections in mice displayed intense gelatinolytic bands localized at 105, 92, 85, 72, and 64–62 kd. Figure 2C (right panel) is representative of both groups of mice in experiment 3; the primary tumors from mice with metastases did not display higher levels of gelatinases than the nonmetastatic group (data not shown). The 105-kd band is consistent with mouse latent gelatinase B; human latent gelatinase B and activated mouse gelatinase B migrates at ~92 kd.[28] The 72-kd and 62-kd gelatinolytic bands could represent human or mouse latent and activated gelatinase A, respectively. Tumor extracts from GFP alone-injected mice revealed weaker gelatinolytic bands (with minimal activated gelatinolytic bands) than EMMPRIN/GFP-injected mice.

Histochemistry and in Situ *Hybridization*

Hematoxylin and eosin staining of resected breast masses revealed extensive replacement of normal mammary tissue with carcinoma in tumors originating from mice injected with EMMPRIN/GFP- or GFP cDNA-transfected MDA-MB-436 cells; other than size of the tumor masses, the EMMPRIN-transfected and vector-transfected tumors were indistinguishable by routine staining. Minimal fibrosis and inflammatory cell infiltration were

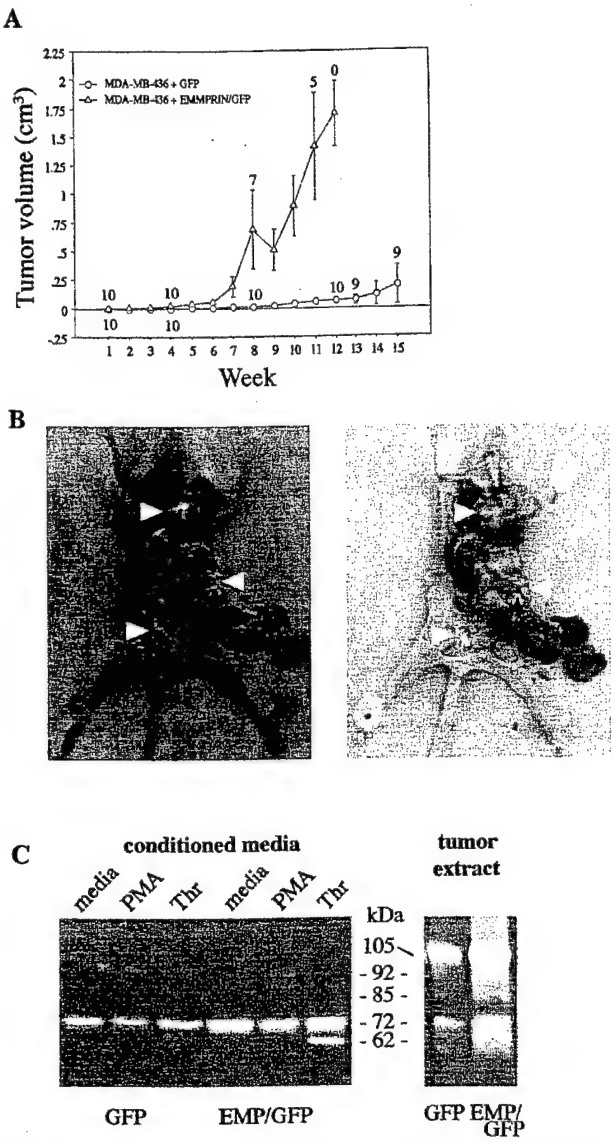


Figure 2. A: MDA-MB-436 breast cancer cells transfected with EMMPRIN/GFP cDNA resulted in enhanced rate of tumor growth after tumor cell implantation into the mammary fat pad of nude mice as compared to GFP-transfected cells. The tumorigenicity of transfected cells was assessed by weekly measurement of tumor size. The data represent the mean ± SE observed in 10 animals in each group injected with 1×10^6 transfected cancer cells. The numbers associated with each symbol refer to the number of mice alive at each time point (ie, at week 8, three mice in the EMMPRIN/GFP group had large tumors and were sacrificed, hence the number seven is listed). **B:** GFP-transfected tumors are readily visible under fluorescent light. EMMPRIN/GFP-transfected MDA-MB-436 breast cancer cells were injected into the mammary tissue of a female NCr *nu/nu* mouse. Eight weeks later, the mouse was sacrificed and extensive green colored metastatic tumors in the peritoneum, liver, spleen, and mediastinum were visible under fluorescent light (**left** photo). The photo on the right demonstrates the same tumors visualized by bright light (**arrowheads** identify tumors). **C:** Comparison of gelatinases secreted by MDA-MB-436 cells cultivated in serum-free medium and extracts of nude mouse tumors. Spent serum-free conditioned medium from primary cells cultivated for 18 hours with vehicle (medium), PMA, and thrombin (**left**) and tumor cell extracts (**right**) were assessed by gelatin substrate zymography. Protein concentration (15 μg/well) of tissue samples was equalized within each group. Conditioned medium and tumor extracts from the EMMPRIN/GFP group displayed more gelatinolytic activity than did the GFP-alone group of mice. The displayed extract from the GFP-alone tumor is from the largest tumor (1.4 cm^3) in this group of mice. The intensity of tumor gelatinolytic activity demonstrated in each group of mice did not correlate with tumor size (data not shown). Molecular weights were calculated using protein standards. The conditioned medium of HT-1080 cells was used to confirm the molecular weight of human gelatinase A and gelatinase B (data not shown).

noted in tumor tissue and surrounding normal-appearing mammary tissue. The sparsity of inflammatory cells in the tumors was confirmed using an antibody (F4/80) that recognizes mouse macrophages, monocytes, and dendritic cells (data not shown).

In situ hybridization of tumor tissue from six mice injected with EMMPRIN/GFP-transfected cells revealed widely distributed, specific staining for EMMPRIN mRNA in cancer cells (Figure 3, panel 2). Surrounding normal-appearing mammary ductal cells and scattered periductal cells also expressed EMMPRIN mRNA (panel 6). Gelatinase A mRNA was found in both cancer cells (panel 3) and the surrounding non-malignant tissue, including normal-appearing mammary ducts and adipose cells (panel 7). There was specific staining for gelatinase B mRNA in the tumor sections (panel 4), but not as widely distributed as gelatinase A. By counting the number of stained cells in serial sections of EMMPRIN cDNA-transfected tumors, the ratio of cells immunotyped as macrophages (F4/80 antibody) *versus* gelatinase B mRNA-expressing cells was ~1:70. Intense staining for gelatinase B was also noted in small aggregates of cells (negative staining for mouse macrophages using F4/80 antibody) scattered around normal-appearing ducts (panel 8). Similar *in situ* hybridization results were found on examination of metastatic tumors in the EMMPRIN/GFP-treated mice (data not shown). Similar results were achieved using either human or mouse gelatinase A and gelatinase B mRNA probes. Specific staining was abolished by pretreatment of tissues with RNase (data not shown). No staining was detected in any of the tumor tissues that were hybridized with EMMPRIN, gelatinase A, or gelatinase B sense probes (data not shown).

In the GFP-alone-transfected tumors (seven mice examined), virtually no EMMPRIN, gelatinase A, or gelatinase B mRNA was identified in the tumor cells or in the surrounding normal-appearing mammary tissue (Figure 3, panels 10 to 12 and 14 to16).

Discussion

The current report describes a direct effect of EMMPRIN expression on tumorigenicity in an animal model. Transfection of EMMPRIN cDNA or EMMPRIN/GFP cDNA into human MDA-MB-436 breast cancer cells resulted in marked enhancement of tumor growth in nude mice after orthotopic injection of tumor cells as compared to injection of vector or GFP alone-transfected tumor cells. High levels of gelatinase A and gelatinase B mRNA expression were demonstrated by *in situ* hybridization in EMMPRIN-transfected tumors as compared to vector- or GFP-transfected tumors. Regardless of the size of the tumors, enhanced gelatinase B and gelatinase A levels were identified in zymograms from extracts of EMMPRIN/GFP-transfected tumors as compared to GFP tumors. The 105-kd gelatinolytic band represents mouse gelatinase B,[29] but the 92-kd band could be either activated mouse gelatinase B or latent human gelatinase B.

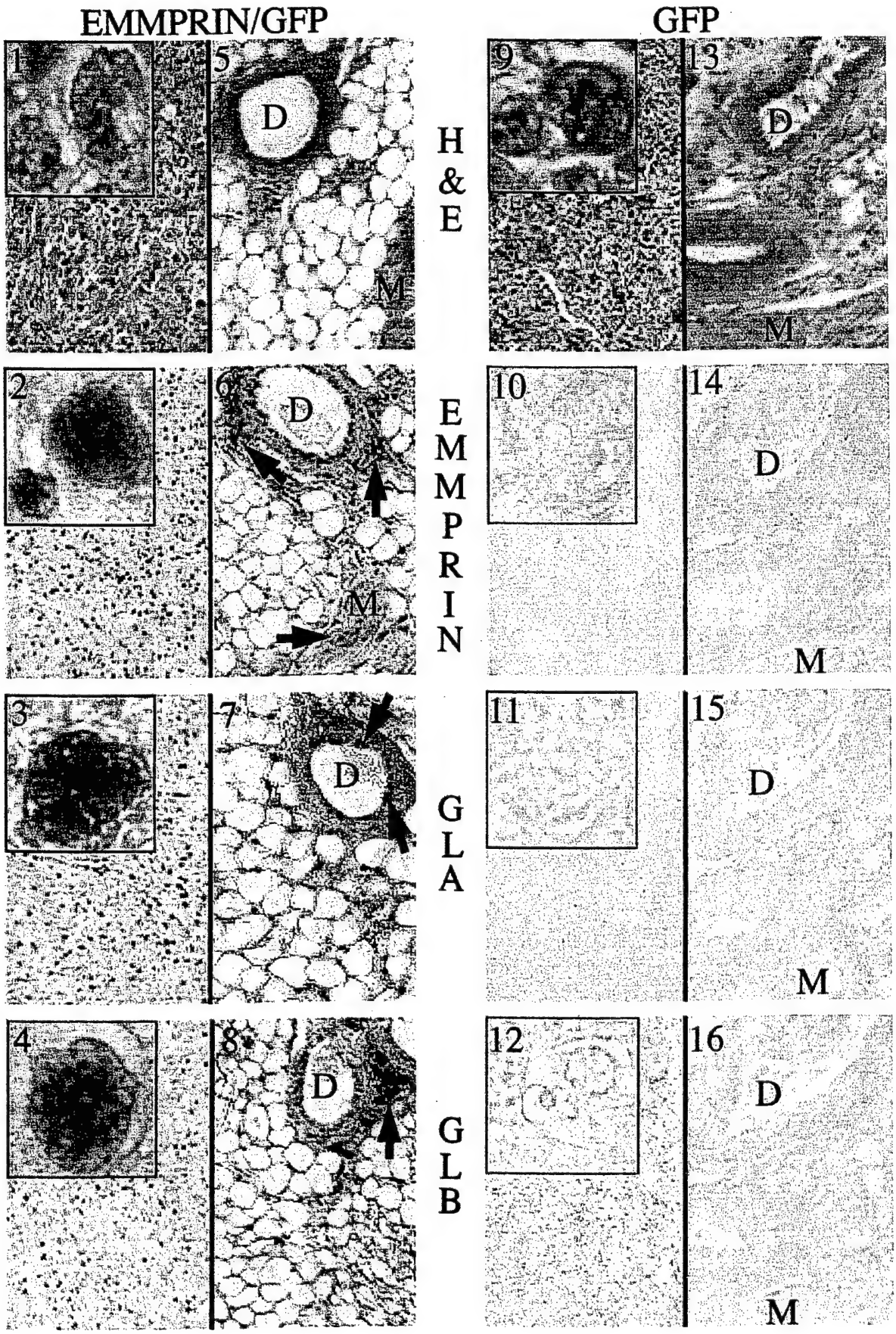
It is noteworthy that human MDA-MB-436 cells propagated *in vitro* readily secreted progelatinase A, but secreted minimal gelatinase B, whereas extracts of tumors removed from nude mice injected with these tumor cells contained higher levels of gelatinase B than gelatinase A. Treatment of these breast cancer cells with thrombin and PMA *in vitro* resulted in increased secretion and activation of progelatinase A and progelatinase B, respectively; this is consistent with the stimulatory effects of these agents described with other types of cells.[25,30] These observations are consistent with the concept that both mouse host cells and transplanted human cancer cells are responsible for the production of gelatinase A and gelatinase B in nude mouse tumors. An association between expression of EMMPRIN and gelatinase B in benign and malignant pigment cell skin lesions in humans has been reported,[31] but a direct stimulatory effect of EMMPRIN on gelatinase B expression or activation has not been previously described. EMMPRIN expression has also been linked to the activation of progelatinase A through a MT-MMP mechanism.[32]

Another important observation in this study was that EMMPRIN-transfected MDA-MB-436 cancer cells secreted higher levels of gelatinase A *in vitro* than vector-transfected cells; this presumably represents autocrine stimulation. These cancer cells also displayed a more invasive phenotype than control transfectants when examined in a modified Boyden chamber (S. Caudroy, M. Polette, B. Nawrocki-Raby, B. Toole, S. Zucker, and P. Birembaut, submitted manuscript).

Our *in situ* hybridization data of tumors transplanted into nude mice differs from *de novo* human breast cancer. Gelatinase A mRNA was identified in EMMPRIN-transfected human cancer cells growing both in nude mice and in surrounding host stromal cells (Figure 3). Previous studies in patients with breast cancer demonstrated the expression of gelatinase A and gelatinase B almost exclusively in peri-tumoral, stromal, and inflammatory cells, respectively.[6,8,33] However, a few reports have described gelatinase B expression in breast,[34] lung,[35] and liver carcinoma cells.[35,36] In comparing experimental cancer models to the human counterpart, it needs to be emphasized that cancer cell lines propagated *in vitro* (eg, MDA-MB-436) that are selected for their invasive properties generally express high levels of gelatinases.[37] Furthermore, by comparison to *in situ* human breast cancers,[38] transplanted human tumors in nude mice demonstrate sparse inflammatory and fibrotic reactions; this represents an important distinction that is often overlooked. These differences between human and animal models of cancer need to be considered in predicting human responses to novel therapies developed in experimental animal models.

A technical aspect of this study that needs explanation relates to quantitative differences in expression of EMMPRIN, gelatinase A, and gelatinase B using different methodologies (Figures 1B, 2C, and 3). It should be noted that the nonradioactive digoxigenin-labeled RNA probes used in this study provide increased resolution and rapid detection of cellular messages, but are less sensitive than autoradiography[39] and substrate zymography.

In one of three sets of experiments, metastasis after orthotopic injection of tumor cells into nude mice oc-


EMMPRIN/GFP
GFP
1
5
D
M
9
13
D
M
H & E
2
6
D
M
10
14
D
M
EMMPRIN
3
7
D
11
15
D
M
GLA
4
8
D
12
16
D
M
GLB

curred more frequently with EMMPRIN-transfected cells than with vector-transfected cells, but the overall rate was low. As we reported previously,[40] EMMPRIN expression did not affect tumor cell proliferation *in vitro*. Based on the established role of EMMPRIN in enhancing MMP synthesis by stromal cells, it would appear that increased degradation of extracellular matrix permits more rapid tumor growth *in vivo*. The higher rate of tumor growth with EMMPRIN-transfected cancer cells and the associated matrix degradation may also occur by favored neoplastic cell survival in a tissue stroma environment initially not permissive for tumor growth. Enhanced extracellular matrix degradation may also release growth factor-like fragments of matrix components, resulting in an indirect effect on cell proliferation.[41] A role for host-derived MMPs in tumor progression and angiogenesis has been supported by studies in gelatinase A-deficient (knockout) mice.[42] In contrast to these findings with EMMPRIN, stromelysin-3 (an MMP with minimal proteolytic activity on extracellular matrix proteins) expression in cancer cells promoted tumor take, but not tumor growth in nude mice.[43] These studies with EMMPRIN reinforce the notion that cancer dissemination is a multistep process and that extracellular matrix degradation contributes to the process but is insufficient in itself to account for tumor metastasis.[44] Continued exploration of genes responsible for the metastatic process is warranted.[45]

Acknowledgments

This article is dedicated to the memory of our friend and colleague, Chitra Biswas, whose career was dedicated to the discovery and exploration of EMMPRIN. Dr. Biswas died in August 1993, but her inspiration continues to guide us in our studies of EMMPRIN. We thank Dr. Serge Lyubsky for his contribution to the histopathological studies and Dr. Philippe Birembaut for helpful discussions.

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Figure 3. *In situ* hybridization of primary tumors from mice injected with EMMPRIN/GFP and GFP-transfected MDA-MB-436 breast cancer cells. Serial sections from tumor tissue (**panels 1–4**, and **9–12**) and surrounding nonmalignant tissue (**panels 5–8** and **13–16**) were examined. **Panels 1** and **9** represent H&E staining of cancer tissues from EMMPRIN/GFP and GFP tumors, respectively; **panels 5** and **13** represent H&E staining of non malignant mammary tissues (tumor cells not identified) adjacent to the primary EMMPRIN/GFP and GFP tumors, respectively. Cells in the primary tumor mass from mice injected with EMMPRIN/GFP-transfected cells revealed widely distributed, specific staining with EMMPRIN, gelatinase A (GLA), and gelatinase B (GLB) antisense riboprobes (**panels 2–4**, respectively). Minimal cell staining for EMMPRIN, gelatinase A, and gelatinase B was seen in cancer cells from GFP-transfected MDA-MB-436 cells (panels 10–12). Nonmalignant tissues adjacent to the primary tumors from EMMPRIN/GFP mice demonstrated focal staining for EMMPRIN, gelatinase A, and gelatinase B in mammary ducts (D) and myocytes (M) (panels 6–8: **arrows** identify EMMPRIN-expressing cells). Nonmalignant tissue from GFP mice revealed no discernable staining for EMMPRIN, gelatinase A, or gelatinase B (**panels 14–16**). The **insets** in **panels 1–4** display higher magnifications of mRNA-stained tumor cells; prominent nuclear staining is noted. Comparable cells (but minimally stained) in the GFP-transfected tumors are demonstrated in **panels 10–12**.

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APPENDIX 2:

Sameshima, T., Nabeshima, K., Toole, B.P., Yokogami, K., Okada, Y., Goya, T., Koono, M., and Wakisaka, S.: Expression of EMMPRIN (CD147), a cell surface inducer of matrix metalloproteinases, in normal human brain and gliomas. Int. J. Cancer **88**:21-27, 2000.

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EXPRESSION OF EMMPRIN (CD147), A CELL SURFACE INDUCER OF MATRIX METALLOPROTEINASES, IN NORMAL HUMAN BRAIN AND GLIOMAS

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EMMPRIN (extracellular matrix metalloproteinase inducer), also called CD147, basigin or M6 in the human, is a member of the immunoglobulin superfamily that is present on the surface of tumor cells and stimulates adjacent fibroblasts to produce matrix metalloproteinases (MMPs). In our study, we investigated expression of EMMPRIN in human normal brain and gliomas, since mouse basigin and chicken HT7, the species homologues of human EMMPRIN, are associated with neuronal interactions and normal blood-brain barrier function, respectively. EMMPRIN expression was detected in all samples of non-neoplastic brain and glioma tissues examined. However, expression levels of EMMPRIN mRNA and protein were significantly higher in gliomas than in non-neoplastic brain. Moreover, levels of mRNA expression and immunohistochemical staining correlated with tumor progression in gliomas: They were highest in the most malignant form of glioma, glioblastoma multiforme, followed by anaplastic astrocytoma and then low-grade astrocytoma. Also, immunolocalization revealed quite different distributions in non-neoplastic brain and glioma: EMMPRIN was demonstrated only in vascular endothelium in non-neoplastic regions of the brain, whereas it was present in tumor cells but not in proliferating blood vessels in malignant gliomas. These data indicate that an MMP inducer molecule EMMPRIN is differently expressed in human normal brain and gliomas and could be associated with astrocytoma progression. Possible mechanisms whereby glioma cell EMMPRIN could influence tumor progression will be discussed. *Int. J. Cancer* 88:21–27, 2000.
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Degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is a crucial step in tissue remodeling and tumor invasion and metastasis (Biswas and Toole, 1987; Liotta *et al.*, 1991). In tumor invasion, not only tumor cells but also host stromal cells, especially fibroblasts, are responsible for MMP production, and the role of tumor cell-fibroblast interactions in regulation of MMP levels in tumors has been demonstrated by several investigators, including ourselves (Biswas, 1982; Dabbous *et al.*, 1983; Goslen *et al.*, 1985; Nabeshima *et al.*, 1994; Himelstein *et al.*, 1994; Ito *et al.*, 1995; Kurogi *et al.*, 1996). Moreover, *in vivo, in situ* hybridization studies have demonstrated that most tumor-associated MMPs, such as interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), stromelysin 3 (MMP-11) and gelatinase A (72-kDa gelatinase, MMP-2), are mainly synthesized in peritumoral fibroblasts rather than in tumor cells themselves in breast, colon, lung, skin and head and neck cancers (Basset *et al.*, 1990; Gray *et al.*, 1992; Pyke *et al.*, 1992; Poulsom *et al.*, 1992, 1993; Noël *et al.*, 1994; Majmudar *et al.*, 1994; Okada *et al.*, 1995; Heppner *et al.*, 1996; Polette *et al.*, 1997). The physiological activators of pro-MMP-2, membrane-type MMP (MT-MMP), are also expressed in the stroma of breast, colon and head and neck cancers (Okada *et al.*, 1995). Thus, not only production but also extracellular activation of MMPs may be aided by stromal cells in tumors, and indeed recent investigations have shown that stromal MMP production promotes tumor progression (Itoh *et al.*, 1998; Noël et al., 1998; Masson *et al.*, 1998).

Extracellular matrix metalloproteinase inducer (EMMPRIN; previously termed tumor cell-derived collagenase stimulatory factor or TCSF), was discovered by the Biswas laboratory via a functional approach and shown to be a surface molecule on tumor cells that stimulates nearby fibroblasts to produce MMP-1, -2 and -3 (Biswas, 1982; Ellis *et al.*, 1989; Prescott *et al.*, 1989; Nabeshima *et al.*, 1991; Kataoka *et al.*, 1993; Biswas *et al.*, 1995; Guo *et al.*, 1997). We have found that EMMPRIN also stimulates production of pro-MMP-2 activators, MT1- and MT2-MMP by fibroblasts (data not shown). The expression levels of EMMPRIN, as determined by immunohistochemistry, are up-regulated in urinary bladder, breast and lung carcinomas compared with their normal counterparts, and EMMPRIN has been implicated in progression and invasion in these tumors in these studies (Muraoka *et al.*, 1993; Polette *et al.*, 1997). The cDNA for human EMMPRIN encodes a 269-amino acid residue polypeptide that includes a signal peptide of 21 amino acid residues, a 185-amino acid extracellular domain consisting of 2 regions characteristic of the immunoglobulin superfamily, followed by a 24-amino acid residue transmembrane domain and a 39-amino acid cytoplasmic domain (Biswas *et al.*, 1995). Recombinant (r)-EMMPRIN isolated from CHO cells transfected with EMMPRIN cDNA was highly glycosylated with a molecular mass of approx. 58 kDa, similar to tumor cell surface EMMPRIN, and successfully stimulated production of MMP-1, -2 and -3 (Guo *et al.*, 1997). Moreover, the EMMPRIN sequence has been found to be identical to that of human basigin (Miyauchi *et al.*, 1991) and human leukocyte activation-associated M6 antigen (Kasinrerk *et al.*, 1992), the species homologue of rat OX-47 antigen (Fossum *et al.*, 1991), mouse basigin (gp42) (Miyauchi *et al.*, 1991) and chicken blood-brain barrier (BBB)-specific HT7 molecule, also known as neurothelin (Seulberger *et al.*, 1990; Schlosshauer and Herzog, 1990), all of which are now called CD147.

Gliomas are the most common human primary brain tumors, and astrocytic tumors comprise the largest subgroup of these tumors (Kleihues, 1993). The most malignant form of the gliomas, glioblastoma multiforme (GBM), may occur *de novo* or may result from progression of low-grade gliomas. For example, astrocytoma may progress to anaplastic astrocytoma, then to GBM. The ability to infiltrate and invade the surrounding normal brain tissue is very characteristic of malignant astrocytic tumors, such as anaplastic astrocytoma and GBM, and degradation of ECM by MMPs is involved in this invasion (Giese and Westphal, 1996). Among MMPs, MMP-2 expression has been shown to correlate most closely with malignant progression of gliomas *in vivo* and with invasive activity of human glioma cells *in vitro* (Nakano *et al.*,

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1995; Sawaya *et al.,* 1996; Uhm *et al.,* 1996; Lampert *et al.,* 1998; Nakada *et al.,* 1999). Moreover, we have found that glioma cell-stromal cell interactions via EMMPRIN regulate MMP-2 levels and its activation *in vitro* (data not shown). Thus, in our study, we investigated EMMPRIN expression levels in human normal brain and gliomas and found that EMMPRIN is up-regulated in gliomas and its expression levels correlate with malignant potential of the tumor.

MATERIAL AND METHODS

Tissues

Human brain glioma tissues and non-neoplastic brain tissues were obtained immediately after surgical removal or autopsy, fresh-frozen in liquid nitrogen and stored at −80°C for RNA and protein extraction. At the same time, portions of the tissues were embedded in OCT compound (Miles, Elkhard, IN), frozen in liquid nitrogen, cut into cryostat sections and stored at −40°C for immunostaining. Standard light microscopic evaluation of each section stained with hematoxylin-eosin was performed for histological diagnosis. The histological grading of gliomas was determined according to the WHO standards (Kleihues *et al.,* 1993).

RNA isolation

Total RNA was extracted from tissues using Trizol (Gibco BRL, Gaithersburg, MD), and poly(A)+RNA was selected with an oligo-dT-Latex (Takara, Shiga, Japan) according to the manufacturer's instructions. Poly(A)+RNA isolation from cultured cells was done using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA), and genomic DNA was isolated using the Dna-Quick DNA extraction kit (Dainippon Seiyaku, Osaka, Japan). Commercially available human whole brain poly(A)+RNA (Clontech, Palo Alto, CA) was also used.

RT-PCR

Poly(A)+RNA (0.1 μg) was reverse transcribed by Superscript reverse transcriptase (Gibco BRL) using an oligo dT primer to prepare cDNA, according to the manufacturer's instructions. The resultant cDNAs were suspended into an aliquot containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 mM dNTPs and 7 mM anti-Taq polymerase monoclonal antibody (MAb) (Clontech). In addition, each sample contained 0.1 pmol of both forward and reverse primers and 2.5 U of Taq polymerase (Takara). PCR was carried out in a thermocycler (Perkin Elmer Cetus, Norwalk, CT) using 5 cycles consisting of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min and 15 sec, followed by 30 cycles consisting of 94°C for 10 sec, 58°C for 30 sec and 72°C for 1 min and 15 sec. Products were analyzed by 2.0% agarose gel electrophoresis.

Two primer pairs (P1 and P2) were used for PCR detection of EMMPRIN. Each pair spanned intron-exon splice sites. The sequences of the forward primers, P1 and P2 sense, correspond to bases 67–83 and 502–521, respectively, and the sequences of reverse primers, P1 and P2 antisense, correspond to bases 251–270 and 782–801, respectively (Biswas *et al.,* 1995). Their product sizes were 204 (P1) and 300 bp (P2). Their amplification of the correct sequences of EMMPRIN was confirmed by the size of PCR product being equal to the expected size and also by nested PCR.

Northern blot analysis

Total RNA (30 μg of each) or Poly(A)+RNA (5 μg of each) was electrophoresed on 1% formaldehyde agarose gel and transblotted onto Hybond-N+ nylon membrane (Amersham, Aylesbury, UK) and RNA was UV-crosslinked onto the membrane. Hybridization was performed in mixed solution of 50% formamide, 5× Denhardt's solution, 25 mM phosphate buffer (pH 6.5), 0.1% SDS, 100 μg/ml of sonicated and heat-denatured salmon sperm DNA and 5× standard saline-citrate (SSC) at 42°C for 16 hr. The blots were washed as follows: 3 times in 0.1% SDS in 1× SSC for 15 min at room temperature and twice in the same solution for 20 min at 65°C. The membranes were autoradiographed with Kodak XR-5 film at −80°C for 6 hr or 18 hr. The full EMMPRIN cDNA (1.6 kb) (Biswas *et al.,* 1995) was used as a probe. For internal control of loading, the blots were subsequently hybridized to a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe (Clontech). The probes were radiolabeled by random priming with ^{32}P-CTP.

For quantification of the RNA blot analysis, the radioactivity of mRNA signals for EMMPRIN was directly measured by a Bioimaging Analyzer, FUJIX BAS2000 system (Fuji Photo Film, Tokyo, Japan) and normalized by division by the corresponding G3PDH mRNA signals. Statistical comparison between groups with different malignant potential was done using Kruskal-Wallis test, and two unpaired samples were compared using Student *t*-test. Significance was set at $p < 0.01$.

Preparation and extraction of membranes

Extraction of EMMPRIN from glioblastoma-cell membranes were performed as described by Ellis *et al.* (1989). Tumor cells were harvested from confluent cultures in 25-cm^2 culture bottles by mechanical scraping and were then suspended and sonicated in 50 mM Tris-HCl (pH 7.4)/0.24 M sucrose. Tissue samples were homogenized in a Polytron in the same Tris-sucrose buffer prior to sonication. The sonicated cell suspension and tissue homogenate were centrifuged at 500g for 1 hr. The membrane pellet was extracted with 0.15 M NaCl/0.5% Nonidet P-40/1 mM EDTA (Nakarai, Kyoto, Japan)/2 mM phenylmethylsulfonyl fluoride (Boehringer Mannheim, Germany)/10 mM Tris-HCl (pH 8.2), at 4°C overnight. The extract was centrifuged at 100,000g for 1 hr, and the supernatant was kept at 4°C until use. The presence of EMMPRIN in the membrane extract was assayed using immunoblotting.

Immunoblotting

SDS-PAGE of tissue and cell extracts and conditioned medium was performed under reducing conditions using a 5% to 15% gradient separating gel (Biomate, Tokyo, Japan). Aliquots of 3.7 μg of total protein in 20 μl were loaded per lane for each sample. After electrophoresis, the proteins were transferred electrophoretically to Immobilon membrane (Millipore, Bedford, MA). After the non-specific sites were blocked with 5% non-fat dry milk in 0.05% Tween-20/Tris-buffered saline, pH 7.6 (TBS-T), at 37°C for 3 hr, the membrane was incubated overnight at 4°C with mouse MAb to human EMMPRIN (E11F4), human MMP-2 (75-7F7; Fuji Chemical Industries, Takaoka, Japan), MT1-MMP (114-1F2; Fuji Chemical Industries), MT2-MMP (162-22G5; Fuji Chemical Industries) or MT3-MMP (117-4E1; Fuji Chemical Industries) dissolved in TBS-T containing 1% BSA. The membrane was washed 3 times with TBS-T and was incubated for 1 hr with peroxidase-conjugated goat anti-mouse IgG. The color was developed with chemiluminescence reagents (DuPont NEN, Boston, MA) according to the manufacturer's instructions. The bands on the film were subjected to image analysis (Adobe Photoshop, Adobe Systems, Mountain View, CA). Statistical analysis was done using Student *t*-test and Kruskal-Wallis test as described above.

Immunohistochemistry

Immunohistochemical staining of frozen sections was performed as described (Muraoka *et al.,* 1993) with minor modifications. Briefly, frozen tissue sections were fixed in acetone at −40°C for 5 min. After endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 30 min, sections were incubated with normal rabbit serum (1:5; DAKO, Glostrup, Denmark) for 1 hr at 37°C. After blocking with normal serum, the sections were incubated with anti-EMMPRIN MAb (E11F4) (Ellis *et al.,* 1989) overnight at 4°C. The sections were then washed in PBS and incubated with biotinylated anti-mouse IgG (1:10; DAKO) for 10 min at room temperature, followed by streptavidin conjugated to horseradish peroxidase (DAKO) for 10

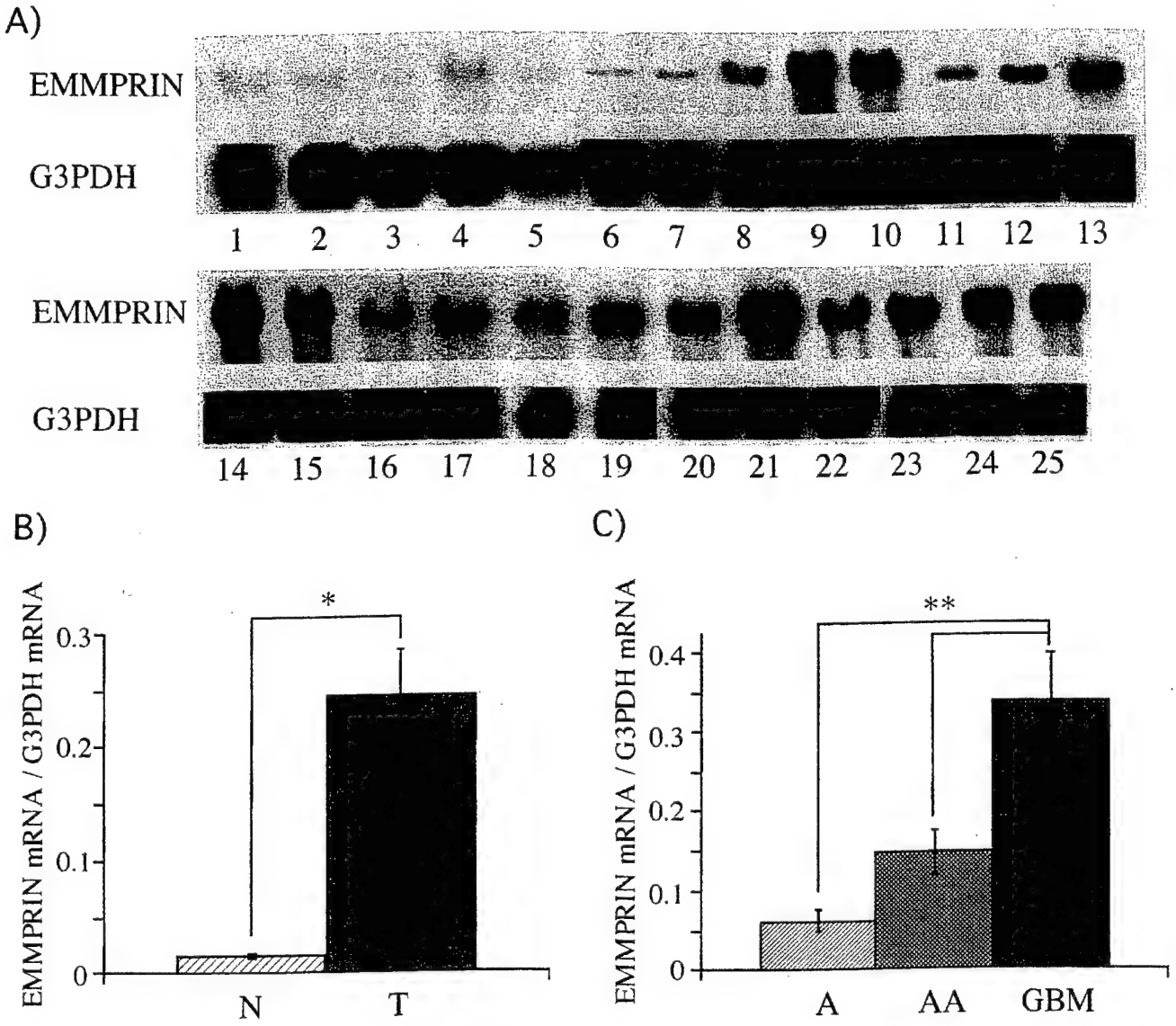

FIGURE 1 – (*a*) Northern blot analysis of EMMPRIN mRNA in human non-neoplastic brain and glioma tissues. Poly (A)+ RNA (5 μg) from each cell line was applied to the gel, then the blot was probed sequentially with EMMPRIN cDNA (indicated by "EMMPRIN") and G3PDH cDNA (indicated by "G3PDH") and autoradiographed for 6 hr. Lanes 1–5, non-neoplastic brain; lane 6, pilocytic astrocytoma; lanes 7–8, low-grade astrocytoma; lanes 9–13, anaplastic astrocytoma; and lanes 14–25, GBM. (*b, c*) The quantification of EMMPRIN mRNA levels, shown as a ratio of the radioactivity of the 1.7-kb EMMPRIN band in *a* to that of the corresponding G3PDH band. N, non-neoplastic brain tissues (n = 5); T, all astrocytic tumors (n = 20); A, astrocytoma (n = 3); AA, anaplastic astrocytoma (n = 5); GBM, glioblastoma multiforme (n = 12); $*p < 0.01$ by unpaired Student *t*-test; $**p < 0.005$ by Kruskal-Wallis test.

min. The reaction was revealed with Metal-3,3′-diaminobenzidine (DAB) (Pierce, Rockford, IL) and counterstained with Mayer's hematoxylin. The immunohistochemical specificity of the antibody was confirmed by 2 types of negative controls: substituting rabbit non-immune IgG for the primary antibody and omitting the primary antibody in the staining protocol.

Vascular endothelial cells, astrocytes and neurons in frozen sections were identified by using antibodies against CD34 (DAKO), GFAP (glial fibrillary acidic protein; DAKO) and neurofilament protein triplet (Signet, Dedham, MA) as primary antibodies, respectively.

Staining results were evaluated semi-quantitatively by 2 independent observers. As described elsewhere (Nabeshima *et al.*, 1997), immunostaining was considered negative if less than 10% of the tumor cells failed to stain. In specimens considered positive, staining of the tumor was quantitated on a scale from 1 to 4 based on the percentage of positive tumor cells. The scale was structured as follows: 1+ = 10% to 25%; 2+ = 25% to 50%; 3+ = 50% to 75%; and 4+ = >75%.

Fisher exact test was used to evaluate the statistical significance of the EMMPRIN protein immunostaining results.

RESULTS

Expression of EMMPRIN mRNA and protein in normal brain and glioma tissues

Pilot experiments using RT-PCR indicated that EMMPRIN mRNA is expressed in normal brain and in gliomas of varying malignancy. To determine whether the amounts of EMMPRIN vary in these tissues, we investigated mRNA levels by Northern blot analysis. All the samples examined expressed EMMPRIN transcripts of approx. 1.7 kb, although the intensity of the signals

varied significantly according to the different sources (Fig. 1*a*). The relative abundance of EMMPRIN mRNA, expressed as a ratio of the radioactivity of the 1.7-kb EMMPRIN band to that of the corresponding G3PDH band, was 0.015 ± 0.002 (n = 5) for non-neoplastic brain tissue and 0.246 ± 0.04 (n = 20) for astrocytic tumors (Fig. 1*b*). Thus, the EMMPRIN mRNA level was up-regulated significantly (approx. 16.4-fold) in astrocytic tumors compared with non-neoplastic tissue ($p < 0.01$ by unpaired *t*-test). Moreover, even within the astrocytic tumors, expression levels increased as the grade of malignancy went up: GBM (0.335 ± 0.06, n = 12) and anaplastic astrocytoma (0.146 ± 0.03, n = 5) expressed approximately 5.5 and 2.4 times more EMMPRIN mRNA, respectively, compared with low-grade astrocytic tumors (0.061 ± 0.01, n = 3). These differences were statistically significant (Fig. 1*c*) ($p < 0.005$ by Kruskal-Wallis test).

To compare amounts of protein in normal and neoplastic brain tissue, we performed immunoblots of tissues with the MAb E11F4. In each case, a doublet at 42 and 40 kDa was detected (Fig. 2*a*). T24 bladder carcinoma cell extracts, used as a positive control, showed a broad band around 50 kDa as described (Muraoka *et al.*, 1993). EMMPRIN protein was also expressed as a doublet in LX-1 human lung carcinoma cells, but the molecular weights in this case were 58 and 54 kDa (Nabeshima *et al.*, 1991). These differences in molecular weight of EMMPRIN are mainly due to varying extents of glycosylation since the protein backbone of EMMPRIN corresponds to an approximate molecular weight of 27 kDa (Biswas *et al.*, 1995). Although previous results suggest that glycosylation may be important for functional activity (Guo *et al.*, 1997), the significance of differing extents of glycosylation is not known. We have shown in other studies that glioma cell EMMPRIN stimulates MMP production in brain-derived fibroblasts (data not shown). Semi-quantitative analysis of the immunoreactive bands in Figure 2 revealed that the EMMPRIN protein was expressed at significantly higher levels (approx. 2.7 times more) in astrocytic tumors compared with non-neoplastic brain tissues (Fig. 2*b*) ($p < 0.005$ by unpaired *t*-test). Correlation between the protein levels and tumor progression could not be determined statistically in this immunoblot study because of the small number of cases.

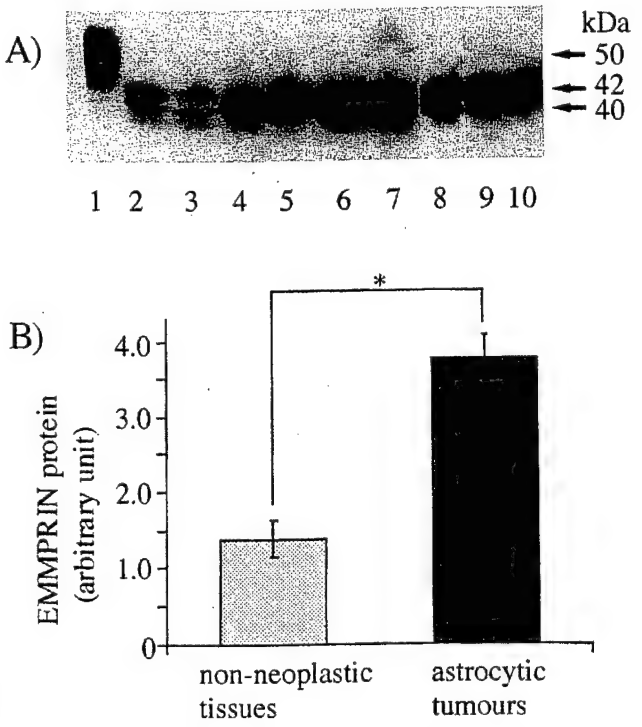

FIGURE 2 – (*a*) Immunoblot analysis of EMMPRIN protein expressed in non-neoplastic and neoplastic brain tissues. Cell and tissue extracts (aliquots of 3.7 μg of total protein in 20 μl) were subjected to immunoblotting with MAb (E11F4) to EMMPRIN. Lane 1, cultured T24 urinary bladder carcinoma cells as positive control; lanes 2–4, non-neoplastic brain; lane 5, low-grade astrocytoma; lane 6, anaplastic astrocytoma; lanes 7–10, GBM. (*b*) Semi-quantitative comparison of EMMPRIN protein expression in non-neoplastic tissues and all astrocytic tumors. The bands on the film in *a* were subjected to image analysis as described. *$p < 0.005$ by unpaired Student *t*-test

Immunohistochemical localization of EMMPRIN

Since EMMPRIN expression was elevated in glioma tissues as compared with normal brain tissue, we examined localization of EMMPRIN in these tissues by immunohistochemistry. In non-neoplastic portions of the brain, EMMPRIN immunostaining was demonstrated only in vascular endothelial cells as cytoplasmic and membrane staining (Fig. 3*a*), which was not present in negative controls with non-immune IgG (Fig. 3*b*). On the contrary, in GBM the tumor cells were stained positively, and proliferating blood vessels were negative (Fig. 3*e*). The tumor cells showed strong positivity with accentuation along the cell membrane. GBM cells at the invasion front were also positive for EMMPRIN (Fig. 3*f*). In low-grade astrocytomas, most of the tumor cells were negative for EMMPRIN, with only vascular endothelial cells being positive (Fig. 3*c*). In anaplastic astrocytomas, varying percentages of the tumor cells were positive (Fig. 3*d*), and some endothelial cells were positive while the others were negative. The regions containing EMMPRIN-positive endothelium did not show Gd-enhancement on magnetic resonance imaging (MRI), suggesting the presence of normally functioning BBB, whereas GBM portions with EMMPRIN-negative proliferating blood vessels demonstrated intense Gd-enhancement, indicating loss of normal BBB function (data not shown). Table I summarizes the results of EMMPRIN immunostaining in glial cells. Expression levels in normal brain and low-grade astrocytomas were <1+, whereas expression levels of ≥3+ were noted in 25% and 100% ($p < 0.001$ by Fisher exact test) of anaplastic astrocytomas and GBM, respectively, indicating a clear relationship between EMMPRIN protein staining and higher grade astrocytic tumors.

DISCUSSION

In our study, we have demonstrated that expression levels of EMMPRIN mRNA and protein are higher in astrocytic tumors than in non-neoplastic brain tissues. Moreover, EMMPRIN immunostaining and mRNA levels are significantly higher in malignant astrocytic tumors than in low-grade astrocytomas, indicating that EMMPRIN expression in the brain could be associated with astrocytoma progression.

EMMPRIN is known as a stimulator of stromal MMP production, and MMPs that can be stimulated by EMMPRIN include MMP-1, -2, -3 (Ellis *et al.*, 1989; Prescott *et al.*, 1989; Kataoka *et al.*, 1993), MT1-MMP and MT2-MMP (data not shown). Among these MMPs, expression of MMP-2 and its activators, MT1-MMP and MT2-MMP, has been reported to correlate with the malignant progression of gliomas *in vivo* (Nakano *et al.*, 1995; Sawaya *et al.*, 1996; Yamamoto *et al.*, 1996; Lampert *et al.*, 1998; Nakada *et al.*, 1999). Activation ratio of MMP-2 also correlates with the glioma progression (Nakada *et al.*, 1999). We have recently shown *in vitro* that EMMPRIN expressed on glioblastoma cells stimulates production of pro-MMP-2 and MT1- and MT2-MMP by brain-derived fibroblasts and, consequently, activation of pro-MMP-2 (data not shown). Thus EMMPRIN might be involved in the increased expression of MMP-2, MT1-MMP and MT2-MMP in malignant gliomas *in vivo*, and EMMPRIN expressed on glioma cells may facilitate gliomal invasion via stimulating production and activation of MMP-2 by stromal cells.

In the brain, both in adults and infants, fibroblastic stromal cells, which are target cells for EMMPRIN, are present in blood vessels outside the endothelium (Zhang *et al.*, 1997). These fibroblasts can

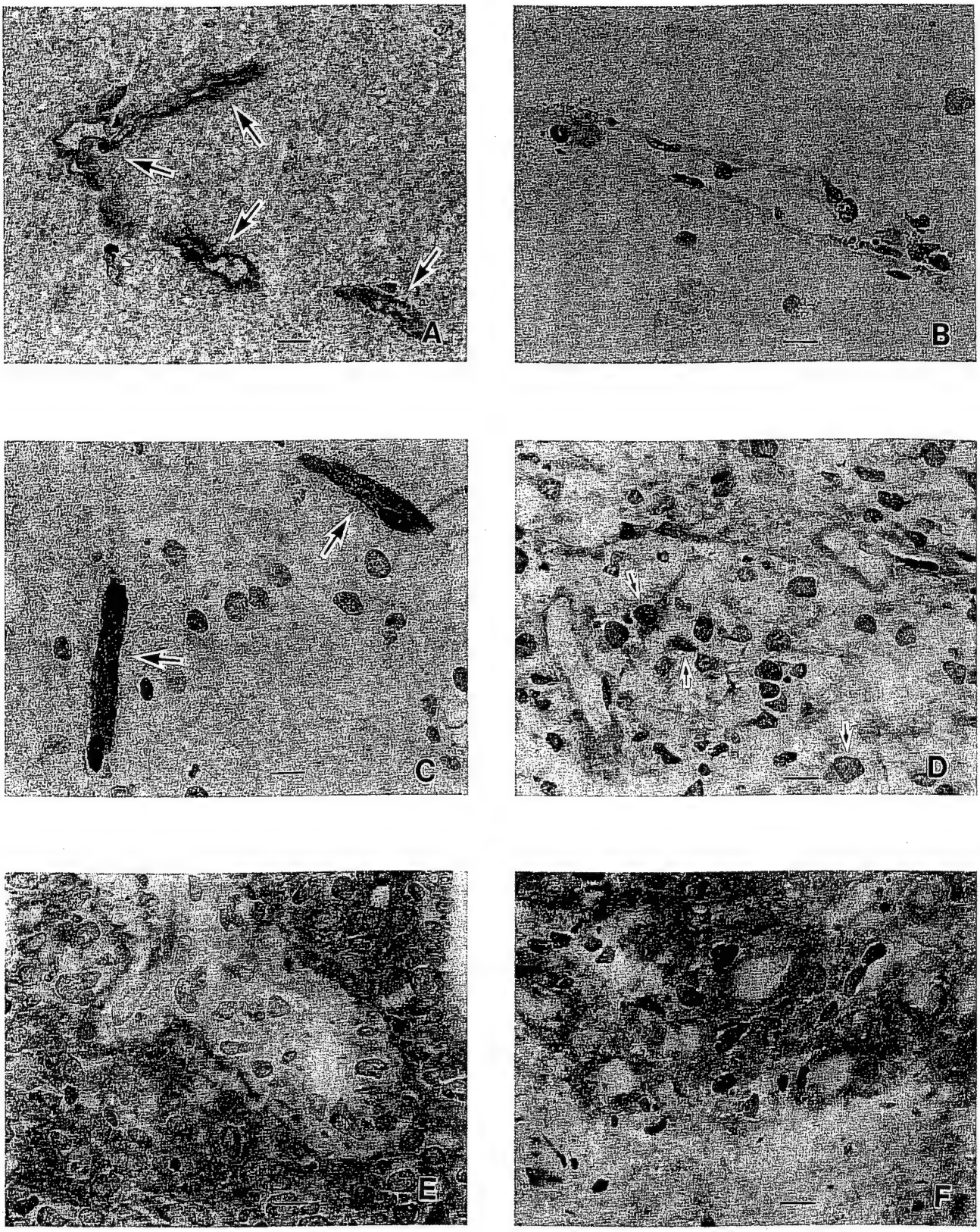

FIGURE 3 – Immunohistochemical demonstration of EMMPRIN protein in normal (*a, b*) and glioma (*c–f*) tissues. (*a*) In the normal brain, vascular endothelial cells (arrows) show strong positive staining, while astrocytes stain negatively. (*b*) Negative control with non-immune mouse IgG, indicating loss of immunostaining of vascular endothelial cells. (*c*) In low-grade astrocytoma, tumor cells stain negatively, with vascular endothelial cells (arrows) being positive. (*d*) In anaplastic astrocytoma, some tumor cells (arrows) stain positively while others stain negatively. Endothelial cells stain very weakly. (*e*) In the center of GBM, tumor cells show diffuse cytoplasmic staining with accentuation along the cell membrane, whereas proliferating blood vessels with thickened layers of cells stain negatively. (*f*) The invasion front of GBM consists of infiltrating EMMPRIN-positive, pleomorphic tumor cells. Scale bar = 10 μm.

TABLE I – IMMUNOHISTOCHEMICAL STAINING LEVEL OF EMMPRIN IN NORMAL BRAIN AND ASTROCYTIC TUMORS

Grade[1]	No. of cases	Level of expression of EMMPRIN					Rate of 3 to 4+ (%)
		–	1+	2+	3+	4+	
Non-neoplastic cerebral tissue[2]	12	12	0	0	0	0	0
Astrocytoma[3]	5	5	0	0	0	0	0
Anaplastic astrocytoma	4	2	1	0	1	0	25
Glioblastoma multiforme	9	0	0	0	2	7	100

[1]Histological diagnosis based on the World Health Organization classification system.–[2]EMMPRIN immunostaining only in glial cells is estimated here.–[3]One case of pilocytic astrocytoma is included.

proliferate and take part in the formation of granulation tissue together with capillaries in response to tissue injuries such as bacterial infection (Gray and Nordmann, 1997). Additionally, blood vessels are the structures in brain that contain ECM proteins known to be substrates for MMPs, such as laminin, type IV collagen, fibronectin and vitronectin (Giese and Westphal, 1996). These proteins are arranged together to form vascular basement membranes (BMs). The intracortical perivascular space, called the Virchow-Robin space, contains another true BM, the glial limitans externa, which exists beneath the pial cells and separates the astrocytic foot processes from the pial cells (Giese and Westphal, 1996). This perivascular space is one of the routes of glioma cell spread, although the white matter fiber pathways are the predominant one (Burger and Scheithauer, 1994). However, penetration of BM and consequent invasion into the vessel lumen rarely occur *in vivo*. In this light, malignant glioma cells may require modification of the surface structures of vascular BMs and the glial limitans externa such that they become more favorable for tumor spread rather than a mechanism for penetration of these structures. Fibroblast MMPs stimulated by glioma cell EMMPRIN might be involved in this type of remodeling of BMs and facilitate the perivascular spread of glioma cells.

A second mechanism whereby gliomal cell EMMPRIN might influence tumor progression is via stimulation of angiogenesis, a characteristic feature of GBM. MMP-2 (Itoh *et al.,* 1998) and MT1-MMP (Hiraoka *et al.,* 1998) have been implicated in angiogenesis. Thus EMMPRIN-stimulated production of these enzymes by fibroblasts in perivascular regions may contribute to tumor angiogenesis. In addition, it has been shown recently that EMMPRIN stimulates endothelial cell production of several MMPs (S. Zucker, M. Drews, C. Conner, J. Cao and B. Toole, unpublished results), which may be directly involved in endothelial cell migration during tumor angiogenesis.

In non-neoplastic portions of the brain, immunostaining of EMMPRIN is confined to the vascular endothelial cells. Moreover, EMMPRIN-positive endothelial cells are confined to the brain: EMMPRIN could not be detected in vascular endothelial cells by immunostaining of cryostat sections of various tissues outside the brain as far as we have examined (data not shown). This immunolocalization is very similar to that of HT7/neurothelin, the species homologue of EMMPRIN in the chicken. HT7/neurothelin is exclusively expressed on endothelial cells of the central nervous system but not on systemic endothelial cells (Seulberger *et al.,* 1990; Schlosshauer and Herzog, 1990). The molecular weight of HT7/neurothelin is approximately 43 kDa (Schlosshauer and Herzog, 1990), which is almost the same as that of EMMPRIN expressed in human brain and gliomas in our study. Endothelial cells in the circumventricular organs that lack BBB, such as the pituitary, median eminence, subfornical organ, pineal gland and area postrema, were negative for HT7/neurothelin (Albrecht *et al.,* 1990). These observations suggest a correlation between BBB function and expression of HT7/neurothelin. We also found that, in the case of malignant gliomas, endothelial cells obtained from intratumoral areas showing Gd-enhancement on MRI, which indicates loss of BBB function, are negative for human EMMPRIN, whereas those from the peritumoral area showing no Gd-enhancement are EMMPRIN-positive (data not shown). Thus, in the normal human brain, EMMPRIN may also be closely related to BBB function, but its specific function in this system is not yet understood. Once EMMPRIN is over-expressed aberrantly in glioma cells, however, it may cause exaggerated remodeling of ECM via interaction with fibroblasts and endothelial cells, which consequently facilitates tumor cell infiltration and tumor angiogenesis. Contact of EMMPRIN-expressing cells with stromal cells might be a key event, since normal EMMPRIN-expressing endothelial cells are separated from the outer stromal cells by the basement membrane.

Our study suggests that interruption of tumor cell-fibroblast (or other stromal cell) interaction could also be a target of anti-invasion therapy in gliomas. Demonstration of the involvement, *in vivo,* of EMMPRIN in the stimulation of production of MMPs essential to glioma progression would be necessary to confirm this possibility.

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APPENDIX 3:

Sameshima, T., Nabeshima, K., Toole, B.P., Yokogami, K., Okada, Y., Goya, T., Koono, M., and Wakisaka, S.: Gliomal cell extracellular matrix metalloproteinase inducer (EMMPRIN) (CD147) stimulates production of membrane-type matrix metalloproteinases and activation of gelatinase A in co-cultures with brain-derived fibroblasts. Cancer Lett. **157**: 177-184, 2000.

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Glioma cell extracellular matrix metalloproteinase inducer (EMMPRIN) (CD147) stimulates production of membrane-type matrix metalloproteinases and activated gelatinase A in co-cultures with brain-derived fibroblasts

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Abstract

Extracellular matrix metalloproteinase inducer (EMMPRIN) also called CD147, basigin or M6 in the human is a member of the immunoglobulin superfamily that is enriched on the surface of tumor cells and stimulates adjacent stromal cells to produce several matrix metalloproteinases (MMPs). In this study, we have demonstrated that coculturing of EMMPRIN-expressing human glioblastoma multiforme cells (U251) with brain-derived human fibroblasts not only stimulates production, but also activation of pro-gelatinase A (proMMP-2), an enzyme that is enriched in malignant gliomas and most likely crucial to tumor progression. Production of membrane types 1 and 2-MMPs (MT1-MMP and MT2-MMP), which are activators of proMMP-2, was also stimulated in these cocultures. Stimulation of MMP-2, MT1-MMP and MT2-MMP production was inhibited by anti-EMMPRIN monoclonal antibody in a dose-dependent manner. Thus, we have shown, for the first time, that EMMPRIN causes increased expression of MT1-MMP and MT2-MMP, as well as increased production and activation of MMP-2.

Keywords: Matrix metalloproteinases; Gelatinase A; Membrane-type matrix metalloproteinases; Extracellular matrix metalloproteinase inducer/basigin; Glioma

1. Introduction

Degradation of the extracellular matrix by matrix metalloproteinases (MMP) is a crucial step in tissue remodeling, tumor invasion and metastasis [1,2]. In situ hybridization studies have demonstrated that, in vivo, most tumor-associated MMPs, such as interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), stromelysin-3 (MMP-11), and gelatinase A (72 kDa gelatinase, MMP-2), are mainly synthesized by peritumoral stromal cells rather than by tumor cells themselves, e.g. in breast, colon, lung, skin and head and neck cancers [3–7]. Recent investigations have shown

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that stromal production of MMPs promotes tumor progression [8–10]. The physiological activator of proMMP-2, membrane-type MMP (MT-MMP), is also expressed in the stroma of several cancers [11]. Thus, not only production but also activation of MMPs may be aided by stromal cells in tumors.

Extracellular matrix metalloproteinase inducer (EMMPRIN), previously termed tumor cell-derived collagenase stimulatory factor or (TCSF) is a highly glycosylated, transmembrane protein of ~55 kDa with an ectodomain consisting of two regions characteristic of the immunoglobulin superfamily [12]. The amino acid sequence of EMMPRIN is identical to that of human basigin [13] and human leukocyte activation-associated M6 antigen [14]. EMMPRIN is enriched on the surface of tumor cells and stimulates adjacent fibroblasts to produce elevated levels of MMP-2, interstitial collagenase and stromelysin-1 [7,12,15–18]. Expression of EMMPRIN is upregulated in several types of human cancers, compared with their normal counterparts, and correlates with tumor progression and invasion in these cancers [6,19]. The species homologue of EMMPRIN includes mouse basigin/gp42 [13] and chicken 5A11/HT7/neurothelin [20–22], and these are expressed in the brain.

Gliomas are the most common human primary brain tumors, and astrocytic tumors comprise the largest subgroup of these tumors [23]. The most malignant form of gliomas, glioblastoma multiforme (GBM), invades surrounding normal brain tissue, and MMPs are involved in this invasion [24]. Among MMPs, MMP-2 expression and activation have been shown to correlate closely with malignant progression of gliomas in vivo and with invasive activity of human glioma cells in vitro [25–29]. MMP-2 forms a complex with and is activated by MT-MMPs at the cell surface [30,31], and MT-MMPs are also associated with glioma progression [29,32]. However, the role of tumor cell-stromal cell interactions, and specifically EMMPRIN, in regulation of MMP levels in gliomas has never been explored, and there are no reports of the effect of EMMPRIN on MT-MMP expression in any system. Thus, in this study, we investigated the potential role of glioma cell EMMPRIN in these events using in vitro cocultures of human GBM cells (U251) with brain-derived fibroblasts. The results show, for the first time, EMMPRIN-dependent stimulation of fibroblast production of proMMP-2 and the MMP-2 activators, MT1-MMP and MT2-MMP, as well as stimulation of proMMP-2 activation.

2. Materials and methods

2.1. Cell culture

Human adult brain-derived fibroblasts, MBT-3, were obtained from a lung adenocarcinoma metastasis to the cerebellum. Embryonic human brain-derived fibroblasts, FLOW3000, were purchased from Human Science Research Resources Bank (Osaka, Japan). The GBM cell line, U251, and a human bladder-cancer cell line, T24, were obtained from the RIKEN cell bank (Tsukuba, Japan). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G and 100 g/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C.

Co-culture experiments were done as previously described [33]. Briefly, cultures containing either MBT-3 fibroblasts, U251 GBM cells or both, were established in 20 mm diameter wells containing 1.0 ml growth medium. In some experiments the MBT-3 cells were replaced by FLOW3000 fibroblasts. A fixed number (1.0×10^5) of fibroblasts was incubated with increasing numbers (0.1, 0.5 and 1.0×10^5) of U251 cells. The cells were allowed to attach for 24 h at 37°C in a humid atmosphere of 5% CO_2 and 95% air, after which their media were replaced with fresh serum-free DMEM containing 0.2% lactalbumin hydrolysate (0.5 ml/well) prior to beginning the experiment. Each experimental condition was done in duplicate wells. Culture fluids were replaced with fresh serum-free DMEM at day 3, and harvested at day 6. The harvested media were used for zymography and immunoblotting.

For the co-culture experiments employing the monoclonal antibody, E11F4 [15], the U251 cells were preincubated with E11F4 before coculturing with fibroblasts. Typically $0.1–0.25 \times 10^5$ U251 cells were incubated at 37°C for 45 min with 200 μl of E11F4, after which the whole mixture was added to the fibroblasts (1×10^5) and allowed to attach for 24 h at 37 °C as described above. Then, their media were replaced with serum-free DMEM containing varying

amounts of serum-free E11F4. This replacement was done again at day 3, and culture fluids were harvested at day 6.

To detect MT1-MMP and MT2-MMP production in the coculture experiments, the cells were harvested at 6 days and lysed by boiling for 5 min in 2% sodium dodecyl sulfate (SDS), 0.1 M dithiothreithol/1 M Tris–HCl (pH 6.8), followed by centrifugation at 11 000 × *g* for 5 min. These SDS extracts were subjected to immunoblotting.

2.2. Zymography

Gelatinolytic activities in conditioned media were demonstrated using gelatin as a substrate as described [34]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under non-reducing conditions using a 9% separating gel containing 1 mg/ml gelatin. After electrophoresis, the gel was shaken gently in detergent buffer (5 mM $CaCl_2$/2.5% Triton X-100/50 mM Tris–HCl, pH 7.6) at room temperature for 60 min to remove SDS, and then incubated in reaction buffer (0.15 M NaCl/10 mM $CaCl_2$/0.02% NaN_3/50 mM Tris–HCl, pH 7.6) at 37°C for 40 h followed by staining with 2.5% Coomassie brilliant blue in 30% methanol and 10% acetate. Enzyme activity was detected as a clear band on the resulting blue background of undigested gelatin. Photos of the gels were subjected to image analysis in order to quantitate relative activities (Adobe Photoshop, Adobe Systems, Mountain View, CA).

2.3. Preparation and extraction of EMMPRIN from cell membranes

Extraction of EMMPRIN from cell membranes was performed as described by Ellis et al. [15]. Cells were harvested from confluent cultures in 25-cm^2 culture bottles by mechanical scraping and were then suspended and sonicated in 50 mM Tris–HCl (pH 7.4)/0.24 M sucrose. The sonicated cell suspension was centrifuged at 500 × *g* for 20 min and the supernatant was centrifuged at 100 000 × *g* for 1 h to pellet the membranes. The membrane pellet was extracted with 0.15 M NaCl/0.5% Nonidet P-40/1 mM ethylenediaminetetra-acetic acid (EDTA) (Nakarai, Kyoto, Japan)/2 mM phenylmethylsulfonyl fluoride (Boehringer Mannheim, Germany)/10 mM Tris–HCl (pH 8.2), at 4°C overnight. The extract was centrifuged at 100 000 × *g* for 1 h and the supernatant was kept at 4°C until use. The presence of EMMPRIN in the membrane extracts was assayed by immunoblotting.

2.4. Immunoblotting

SDS-PAGE of cell extracts and conditioned media was performed under reducing conditions using a 5–15% gradient separating gel (Biomate, Tokyo, Japan). After electrophoresis, the proteins were transferred electrophoretically to Immobilon membrane (Millipore, Bedford, MA). After the non-specific sites were blocked with 5% non-fat dry milk in 0.05% Tween-20/Tris-buffered saline, pH 7.6 (TBS-T), at 37°C for 3 h, the membrane was incubated overnight at 4°C with mouse mAb to human EMMPRIN (E11F4), human MMP-2 (75-7F7, Fuji Chemical Industries, Takaoka, Japan), MT1-MMP (114-1F2, Fuji Chemical Industries), MT2-MMP (162-22G5, Fuji Chemical Industries), or MT3-MMP (117-4E1, Fuji Chemical Industries) dissolved in TBS-T containing 1% bovine serum albumin (BSA). The membrane was washed three times with TBS-T and incubated for 1 h with peroxidase-conjugated goat anti-mouse IgG. The color was developed with chemiluminescence reagents (DuPont NEN, Boston, MA) according to the manufacturer's instructions. To quantitate relative amounts of the MMPs, the bands on the film were subjected to image analysis (Adobe Photoshop).

3. Results

3.1. EMMPRIN expression in human glioblastoma cells

We first determined whether the human glioblastoma cell line, U251, expressed EMMPRIN. A high level of EMMPRIN expression was demonstrated in the U251 cells by immunoblotting (Fig. 1); this level was comparable with T24 bladder carcinoma cells that were previously shown to express high levels of EMMPRIN [19]. A single broad band at approximately 50 kDa was observed in both cell types. However, EMMPRIN protein was not detected in MBT-3 fibroblasts derived from a lung carcinoma metastasis to adult brain; FLOW3000 fibroblasts,

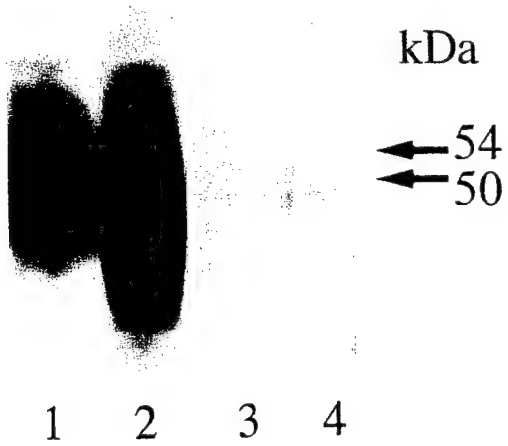

Fig. 1. EMMPRIN expression in U251 glioblastoma cells. Extracts of cell membranes, prepared as described in experimental procedures from similar numbers of cells in each case, were subject to SDS-PAGE and immunoblotting with mAb (E11F4) against EMMPRIN. Lane 1, T24 as positive control; lane 2, U251 cells; lane 3, MBT-3 fibroblasts; lane 4, FLOW3000 fibroblasts.

from normal embryonic brain, showed faint bands at approximately 50 and 54 kDa.

3.2. EMMPRIN-dependent stimulation of MMP-2 production and activation in cocultures of glioma cells and fibroblasts

To assess the potential stimulatory effect of glioblastoma-expressed EMMPRIN on MMP production by fibroblasts in vitro, we ran coculture experiments. A fixed number (1×10^5) of MBT-3 or FLOW3000 brain-derived fibroblasts was incubated with increasing numbers ($0.1–1.0 \times 10^5$) of U251 glioblastoma cells. Fig. 2A shows gelatin zymography of the conditioned media harvested on day 6 of cocultures of U251 glioblastoma cells with MBT-3 fibroblasts. Both MBT-3 and U251 cells exhibited gelatinolytic bands at 68 and 62 kDa. The 68-kDa band corresponds to the molecular mass of the pro-form of MMP-2 in the absence of dithiothreitol and the 62-kDa band corresponds to the activated form [35]. These gelatinolytic activities corresponding to pro- and activated MMP-2 were both stimulated in cocultures compared with individual cultures of each cell type. Moreover, coculturing caused a far greater increase in the activated form of MMP-2 than the pro-form, as much as ~300 vs. ~4-fold, respectively, at a 1:1 ratio of the two cell types (Table 1). Similar results were also obtained in cocultures of U251 tumor cells with FLOW3000 fibroblasts, although the stimulatory effect was a little less than in cocultures of U251 and MBT-3 cells (data not shown). To confirm the above results, we also performed an immunoblot with anti-MMP-2 mAb of the conditioned media harvested on day 6 of culture; only proMMP-2 reacts with this antibody. Again it was found that MMP-2 production was stimulated in

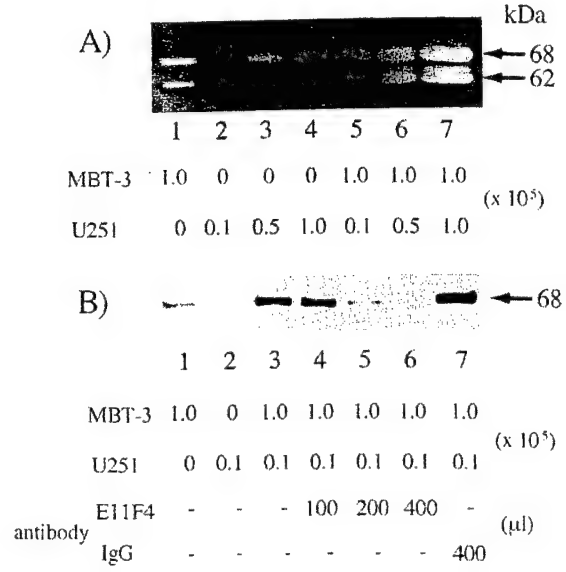

Fig. 2. EMMPRIN-dependent stimulation of MMP-2 production and gelatinolytic activity in cocultures of U251 glioblastoma cells and MBT-3 fibroblasts. (A) Stimulation of MMP-2 gelatinolytic activity in cocultures of U251 and MBT-3 cells. Gelatin zymography was performed with culture media collected at day 6 of culture. Lane 1, MBT-3 alone (1×10^5); lane 2, U251 alone (0.1×10^5); lane 3, U251 alone (0.5×10^5); lane 4, U251 alone (1.0×10^5); lane 5, MBT-3 and U251 (1:0.1); lane 6, MBT-3 and U251 (1:0.5); and lane 7, MBT-3 and U251 (1:1). (B) Reversal of MMP-2 stimulation in cocultures of U251 and MBT-3 cells by anti-EMMPRIN mAb, E11F4. Culture media collected at day 6 of culture were subjected to immunoblotting with anti-MMP-2 mAb. Lane 1, MBT-3 alone (1×10^5); lane 2, U251 alone (0.1×10^5); lane 3, MBT-3 and U251 (1:0.1); lane 4, MBT-3 and U251 (1:0.1) with 100 μl anti-EMMPRIN mAb (E11F4) in a total volume of 500 μl (100/500); lane 5, MBT-3 and U251 (1:0.1) with 200 μl E11F4 (200/500); lane 6, MBT-3 and U251 (1:0.1) with 400 μl E11F4 (400/500); lane 7, MBT-3 and U251 (1:0.1) with 400 μl IgG (400/500).

Table 1
Stimulation of pro- and activated MMP-2 in cocultures of U251 glioma cells and MBT-3 brain-derived fibroblasts

Fibroblasts: glioma cells	Stimulation index (cocultures vs. individual cultures)[a]		
	1:0.1	1:0.5	1:1
Pro-MMP-2	38.1 ± 20.4	2.26 ± 0.55	3.93 ± 0.92
Activated MMP-2 (62 kDA)	20.6 ± 10.0	137 ± 78.7	324 ± 180

[a] Stimulation index is defined as the ratio of MMP-2 in cocultures to the sum of individual cultures of U251 glioma cells and MBT-3 fibroblasts, as measured by densitometry of zymograms from three separate culture experiments. The indices were calculated separately for the pro-MMP-2 and activated MMP-2 bands. Values are mean ±SEM, ($n = 3$).

cocultures compared with individual cultures of MBT-3 and U251 cells (Fig. 2B).

To determine whether EMMPRIN was involved in the above stimulation of MMP-2 activity, we performed coculture experiments in the presence of varying concentrations of activity-blocking anti-EMMPRIN mAb, E11F4 [15]. Stimulation was inhibited by E11F4 in a dose-dependent manner, while control IgG did not cause any inhibition (Fig. 2B).

3.3. EMMPRIN-dependent stimulation of MT1-MMP and MT2-MMP production in cocultures of glioma cells and fibroblasts

Since the activated form of MMP-2 was greatly stimulated by EMMPRIN in the above cocultures, we examined whether EMMPRIN also stimulates production of the activators of MMP-2: MT1-, MT2-, and MT3-MMP [29–31]. Fig. 3 shows an immunoblot with anti-MT1-MMP and anti-MT2-MMP mAbs of extracts of cells harvested as above on day 6 of culture. The 60-kDa band corresponding to the activated form of MT1-MMP was observed, as well as 68 and 62 kDa bands corresponding to the pro-form and activated form, respectively, of MT2-MMP. The level of MT1-MMP was stimulated 10.5(±1.2)-fold ($n = 4$) in cocultures compared with control, i.e. the combined extracts of equivalent numbers of MBT-3 and U251 cells after they had been cultured separately. MT2-MMP was also stimulated in cocultures by 4.0(±1.1)-fold ($n = 4$) compared with control.

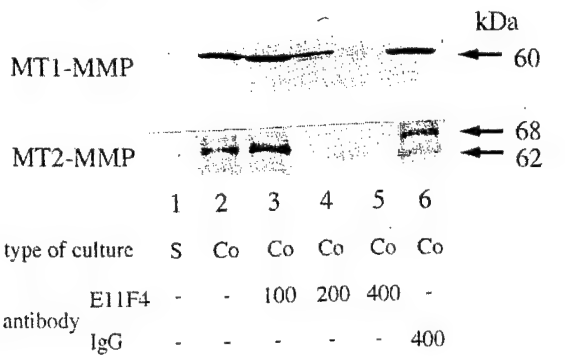

Fig. 3. EMMPRIN-dependent stimulation of MT1-MMP and MT2-MMP production in cocultures of U251 glioblastoma cells and MBT-3 fibroblasts. Extracts of cells collected at day 6 of culture were subjected to immunoblotting with anti-MT1-MMP mAb or anti-MT2-MMP mAb. Lane 1, MBT-3 (1.0×10^5) and U251 (0.25×10^5) cultured separately and extracted together; lane 2, cocultured MBT-3 and U251 (1:0.25); lane 3, cocultured MBT-3 and U251 (1:0.25) with 100 μl anti-EMMPRIN mAb (E11F4) in a total volume of 500 μl (100/500); lane 4, cocultured MBT-3 and U251 (1:0.25) with 200 μl E11F4 (200/500); lane 5, cocultured MBT-3 and U251 (1:0.25) with 400 μl E11F4 (400/500); lane 6, cocultured MBT-3 and U251 (1:0.25) with 400 μl IgG (400/500). S, separate culture; Co, coculture.

These stimulatory effects were inhibited by the anti-EMMPRIN mAb, E11F4 in a dose-dependent manner (Fig. 3). MT3-MMP was not detected in separate cultures or cocultures (data not shown).

4. Discussion

Past studies by several investigators have shown that proMMP-2 is activated by MT-MMPs at the cell surface after binding to a complex containing MT-MMP and TIMP-2 [30,31]. In this study, we have demonstrated that cocultures of GBM cells and brain fibroblasts produce elevated levels of MMP-2, MT1-MMP and MT2-MMP, and give rise to greatly increased activation of MMP-2, as compared with either cell type alone. Furthermore, these effects were shown to be dependent on EMMPRIN present on the surface of the glioma cells. Thus, in this system tumor cell EMMPRIN is required for increased activation as well as production of fibroblast MMP-2. In addition, EMMPRIN stimulates production of the MMP-2 activators, MT1-MMP and MT2-MMP. However, it is yet known whether EMMPRIN stimu-

lates production of each enzyme directly or whether EMMPRIN initiates a cascade of events that leads to their production and action in this coculture system.

MMP-2 expression correlates with the malignant progression of gliomas in vivo [25–29], and MT1-MMP mRNA levels are significantly higher in malignant astrocytomas than in low-grade gliomas and normal brain tissues [28,29,36]. Thus, EMMPRIN expressed on glioma cells may be involved in gliomal invasion via stimulating stromal cell production of MMP-2 and its activation. In support of this proposition, it has been shown recently in other cancer types that stromal production of active MMP-2 promotes tumor progression [8]. In vivo, one of the routes of gliomal cell infiltration is perivascular, a site that contains fibroblasts [37]. Thus gliomal stimulation of these fibroblasts may lead to production and activation of MMP-2, and consequently promotion of invasion. Another mechanism whereby gliomal cell EMMPRIN might influence tumor progression is via stimulation of angiogenesis, a characteristic feature of GBM. MMP-2 [8] and MT1-MMP [38] have been implicated in angiogenesis. Thus, EMMPRIN-stimulated production of these enzymes by fibroblasts in perivascular regions may contribute to tumor angiogenesis. In addition, it has been shown recently that EMMPRIN stimulates production of several MMPs, including MMP-2, by endothelial cells themselves (S. Zucker, M. Hymowitz, R. Mann, C. Conner, J. Cao, B. Toole, manuscript submitted for publication). EMMPRIN-stimulated production and activation of MMP-2 may then be directly involved in endothelial cell remodeling during tumor angiogenesis, thus promoting tumor progression.

A major route of invasion by gliomal cells is along white matter fiber tracts, a pathway that is most likely promoted by MT1-MMP-mediated removal of inhibitory myelin proteins [32]. Thus, in addition to the potential effects of EMMPRIN-stimulated MMP-2 production and activation, EMMPRIN stimulation of MT1-MMP production may promote spreading of gliomal cells along white matter tracts, especially in areas where the GBM has attracted new vessels and associated fibroblasts.

Our study suggests that interruption of tumor cell-fibroblast (or other stromal cell) interaction could be a target of anti-invasion therapy in gliomas. Demonstration of the involvement of EMMPRIN, in vivo, in the stimulation of production of MMPs essential to glioma progression would be necessary to confirm this possibility. This is now under investigation in our laboratories.

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APPENDIX 4:

Guo, H., Li, R., Zucker, S., and Toole, B.P.: EMMPRIN (CD147), an inducer of matrix metalloproteinase synthesis, also binds interstitial collagenase (MMP-1) to the tumor cell surface. Cancer Res. **60**: 888-891, 2000.

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Advances in Brief

EMMPRIN (CD147), an Inducer of Matrix Metalloproteinase Synthesis, Also Binds Interstitial Collagenase to the Tumor Cell Surface[1]

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Abstract

Extracellular matrix metalloproteinase inducer (EMMPRIN), also known as basigin or CD147, is a glycoprotein that is enriched on the surface of tumor cells and stimulates production of several matrix metalloproteinases by adjacent stromal cells. In this study, we have found that EMMPRIN not only stimulates the production of interstitial collagenase (MMP-1) but also forms a complex with MMP-1 at the tumor cell surface. Complex formation was demonstrated by phage display, affinity chromatography, and immunocytochemistry. Presentation of MMP-1 complexed to EMMPRIN at the tumor cell surface may be important in modifying the tumor cell pericellular matrix to promote invasion.

Introduction

MMPs[3] have been implicated in several aspects of tumor progression, including invasion through basement membranes and interstitial matrices, angiogenesis, and tumor cell growth (1–3). Strong support for the involvement of MMPs at some step in tumor progression comes from experiments in which tissue inhibitors of MMPs or synthetic inhibitors of metalloproteinases have been shown to reduce tumor growth and metastasis (4, 5). Over the past several years, it has become increasingly apparent that tumor cells create a pericellular environment in which MMPs and other proteases become concentrated, thus enhancing the ability of tumor cells to invade extracellular matrices (6–8). Previous studies from this laboratory have demonstrated that EMMPRIN, a member of the immunoglobulin superfamily that is enriched on the surface of most tumor cells, stimulates stromal cells to produce elevated levels of several MMPs, including MMP-1 (9–11). We have now found that tumor cell EMMPRIN not only stimulates MMP-1 production by fibroblasts but also binds MMP-1 to the surface of tumor cells, thus adding to the complement of proteases on the tumor cell surface that may promote invasion.

Materials and Methods

Phage Display Library. mRNA was prepared from human fibroblasts with the Oligotex mRNA kit (Qiagen, Valencia, CA) and used for cDNA synthesis with the Directional RH primer cDNA synthesis kit (Novagen, Madison, WI). After second-strand synthesis, the cDNA ends were flushed with T4 DNA polymerase and ligated to *Eco*RI/*Hin*dIII directional linkers. The cDNA was then digested with *Eco*RI and *Hin*dIII and ligated to T7Select1-1b vector arms (Novagen). The ligated DNA was packaged into bacteriophage T7 using the T7Select1-1 Packaging Extract (Novagen). The host strain of bacteria, BLT 5403 (Novagen), was then grown to $A_{600\ nm}$ = 0.8–1.0 and mixed with the packaged cDNA (at a ratio of 10^6 phage/10 ml cells) in LB media containing 50 μg/ml carbenicillin (Novagen). Molten top agarose at 45°C–50°C was added to the phage/host mixture (10:1) and immediately poured onto a 150-mm plate containing LB/carbenicillin medium. The plate was incubated at room temperature overnight until the plaques were nearly confluent. The phage was then eluted by covering the plate with phage extraction buffer [100 mM NaCl, 20 mM Tris, and 6 mM $MgSO_4$ (pH 8.0)] at 4°C overnight. The phage lysate was clarified with chloroform and subjected to screening by biopanning.

Screening of Phage Display Library. Twenty four-well cell culture plates were prepared for biopanning as suggested by the manufacturer (Novagen). The wells were coated with immunopurified EMMPRIN protein (Ref. 12; 1 μg/ml in Tris-buffered saline) at 4°C overnight and washed with Tris-buffered saline five times. Unreacted sites were blocked with 5% blocking reagent overnight at 4°C and washed. In the first round of screening, the phage lysate was applied to the EMMPRIN-coated plate (0.5 ml lysate/well) for 30 min at room temperature. The plate was then washed five times with Tris-buffered saline. The bound phages were eluted by adding 0.5 ml of elution buffer (1% SDS) at room temperature for 20 min. The eluted phages were then added to a culture of the host cells (BLT 5403) in LB media and incubated at 37°C with shaking for 3 h, at which time lysis was observed. The lysed culture was centrifuged, and the supernatant was collected for the next round of biopanning. A total of five rounds of screening was carried out. DNA from the phages isolated during the final round of screening was purified and sequenced using the T7 SelectUp primer (GGAGCTGTCGTATTCCAGTC) and the T7 SelectDown primer (AACCCTCAAGACCCGTTTA; Novagen).

Immunoaffinity and Ligand Affinity Chromatography. EMMPRIN was isolated from extracts of membranes from LX-1 human lung carcinoma cells by immunoaffinity chromatography using E11F4 monoclonal antibody against EMMPRIN immobilized on Sepharose beads, as described previously (12).

For manufacture of the ligand affinity medium, EMMPRIN protein (0.5 mg) was first dissolved in coupling buffer [0.1 M $NaHCO_3$ and 0.5 M NaCl (pH 8.3) containing 0.5% NP40]. The coupling solution was then mixed with CNBr-activated Sepharose 4B gel (Pierce; 0.25 g of dried powder swelled and washed in 1 mM HCl for 30 min) at 4°C. After overnight incubation, the gel was washed three times with 5 ml of coupling buffer, followed by incubation in 0.1 M Tris-HCl (pH 8) for 2 h to block any remaining active groups. Then the gel was washed using three cycles of 0.1 M acetate buffer, 0.5 M NaCl (pH 4), and 0.1 M Tris and 0.5 M NaCl (pH 8). After washing, the gel was resuspended in 5 ml of 10 mM Tris buffer (pH 8.3).

Extracts of human fibroblasts [10^8 cells in 5 ml of 10 mM Tris, 0.15 M NaCl, and 0.5% NP40 (pH 8.3)] were added to the EMMPRIN-coupled gel and incubated at 4°C overnight with rotation. The gel was then washed with 10 mM Tris and 0.15 M NaCl containing 30 mM octyl glucoside until the $A_{280\ nm}$ was less than 0.05. Binding proteins were eluted with 0.1 M glycine buffer (pH 2.5) containing 30 mM octyl glucoside. The eluate was neutralized to pH 7 by the addition of 1 M Tris (pH 9.5) and concentrated for further analysis.

ELISA of MMP-1. MMP-1 protein was measured in the eluates from EMMPRIN-Sepharose and in immunopurified EMMPRIN preparations using a commercial ELISA system (Amersham, Piscataway, NJ) according to the manufacturer's instructions. Briefly, 5 or 10 μl of eluate were added to microtiter plates coated with antibody to MMP-1 and incubated for 2 h at 25°C. The plates were washed with phosphate buffer and incubated with anti-MMP-1 antiserum for 2 h. After washing, the plates were incubated with peroxidase-conjugated secondary antibody for 1 h, and processed for color development and measurement at $A_{450\ nm}$ in a microplate spectrophotometer. The concentration of MMP-1 in the eluate was estimated from a standard curve.

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[3] The abbreviations used are: MMP, matrix metalloproteinase; EMMPRIN, extracellular matrix metalloproteinase inducer; MMP-1, interstitial collagenase; MMP-2, gelatinase A; MT-MMP, membrane-type MMP; LB, Luria-Bertani.

SDS-PAGE, Silver Staining, and Western Blotting. Proteins were dissolved in SDS sample buffer containing 0.1 M DTT and heated at 95°C for 5 min. The samples were then subjected to electrophoresis on 10% SDS polyacrylamide gels. The gels were either stained using the Sterling silver staining system (National Diagnostics, Atlanta, CA) or electroblotted onto nitrocellulose membranes and incubated with antibody against EMMPRIN (E11F4; Ref. 12) or against MMP-1 (Calbiochem, La Jolla, CA) for 1 h at room temperature. The immunoreactive protein bands were detected with horseradish peroxidase-conjugated antimouse IgG and chemiluminescence reagent (New England Nuclear Life Science, Boston, MA).

Immunocytochemistry. LX-1 human lung carcinoma cells were seeded into chamber culture slides and cultured for 48 h at 37°C in 5% CO_2 air. The cells were then washed with PBS, fixed in 1% paraformaldehyde in PBS for 45 min at room temperature, quenched with 0.1 M Tris (pH 7.4), and blocked with 1% BSA, 1% goat serum, and 2% nonfat milk in PBS at room temperature for 1 h. The LX-1 cells were then incubated with monoclonal antibody against MMP-1 (Calbiochem) for 1 h at room temperature, followed by Cy3-conjugated Texas red goat antimouse IgG. The cells were washed with PBS, mounted with coverslips, and then observed and photographed using a Zeiss Axioskop-20 microscope.

Results

Phage Display Reveals MMP-1 as an EMMPRIN-binding Protein. We used the T7Select Phage Display System (Novagen) to identify EMMPRIN-binding protein(s) encoded by a cDNA library prepared from human fibroblasts, as described in "Materials and Methods." In this method, each phage becomes coated with a fusion protein comprised of the phage coat protein and a protein generated from the cDNA library used. Phages coated with putative EMMPRIN-binding protein were selected by repeated panning over 24-well plates coated with EMMPRIN. Five rounds of biopanning were carried out, and the final lysate was used for plaque assay, PCR amplification, and sequencing.

Eight clones were obtained from the procedure described above. All eight of the inserts were of identical size, *i.e.*, 0.8 kb, and were found to have identical sequences corresponding exactly to a portion of the human MMP-1 sequence (Fig. 1).

MMP-1 Binds to EMMPRIN-Sepharose. To confirm the binding of EMMPRIN to fibroblast-produced MMP-1, we performed ligand chromatography over Sepharose conjugated with immunopurified EMMPRIN. Fibroblast extracts were mixed with the EMMPRIN-Sepharose, which was then washed and eluted as described in "Materials and Methods." The eluates were subjected to SDS-PAGE, followed by silver staining. On silver staining, a prominent protein band at ~M_r 55,000 was observed, as well as a weaker band at ~M_r 67,000 (Fig. 2*A*); in some cases a ~M_r 45,000 band could also be seen.

Western blots were also performed on the eluates from EMMPRIN-Sepharose using antibody against human MMP-1. The protein band at ~M_r 55,000 (the approximate size of pro-MMP-1, which is M_r 52,000) reacted with anti-MMP-1 antibody (Fig. 2*B*), confirming our results from the phage display. ELISA measurements also revealed MMP-1 in the eluates from EMMPRIN-Sepharose (data not shown). The identities of the ~M_r 67,000 and ~M_r 45,000 proteins are not yet known.

EMMPRIN Forms a Complex with MMP-1 on the Surface of Tumor Cells. Some tumor cells themselves produce small amounts of MMP-1. Thus, we also determined whether, in addition to binding isolated EMMPRIN protein, MMP-1 forms a complex with EMMPRIN present on the surface of LX-1 human lung carcinoma cells. We immunopurified EMMPRIN from extracts of LX-1 cell membranes using monoclonal antibody E11F4 covalently bound to Sepharose beads and tested whether MMP-1 was present in the eluted EMMPRIN preparation. Fig. 3 shows a Western blot of such an

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                1                                                        55
5' sequence:    GGGAGAAGTGATGTTCTTTAAAGACAGATTCTACATGCGCACAAATCCCTTCTAC
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human MMP-1:    GGGAGAAGTGATGTTCTTTAAAGACAGATTCTACATGCGCACAAATCCCTTCTAC
                941                                                     995

                56                                                      110
                CCGGAAGTTGAGCTCAATTTCATTTCTGTTTTCTGGCCACAACTGCCAAATGGGC
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
                CCGGAAGTTGAGCTCAATTTCATTTCTGTTTTCTGGCCACAACTGCCAAATGGGC
                996                                                     1050

                111                                                     165
                TTGAAGCTGCTTACGAATTTGCCGACAGAGATGAAGTCCGGTTTTTCAAAGGGAA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
                TTGAAGCTGCTTACGAATTTGCCGACAGAGATGAAGTCCGGTTTTTCAAAGGGAA
                1051                                                    1105


                1                                                        55
3' sequence:    GGAGTGCCTGATGTGGCTCAGTTTGTCCTCACTGAGGGGAACCCTCGCTGGGAGC
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human MMP-1:    GGAGTGCCTGATGTGGCTCAGTTTGTCCTCACTGAGGGGAACCCTCGCTGGGAGC
                343                                                     397

                56                                                      110
                AAACACATCTGACCTACAGGATTGAAAATTACACGCCAGATTTGCCAAGAGCAGA
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
                AAACACATCTGACCTACAGGATTGAAAATTACACGCCAGATTTGCCAAGAGCAGA
                398                                                     452

                111                 131
                TGTGGACCATGCCATTGAGAA
                |||||||||||||||||||||
                TGTGGACCATGCCATTGAGAA
                453                 473
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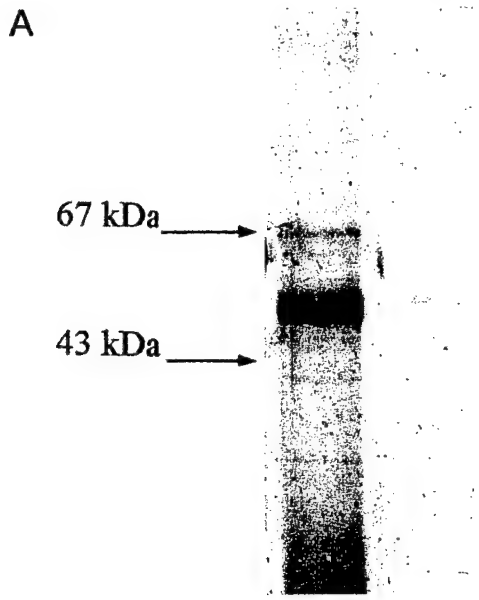
Fig. 1. Comparison of partial sequences of cDNA isolated by phage display with that of human MMP-1 cDNA. The 5′ and 3′ sequences of one of the partial cDNAs obtained are given. Eight cDNA clones were isolated after biopanning of phages on EMMPRIN; all eight clones had identical sequences.



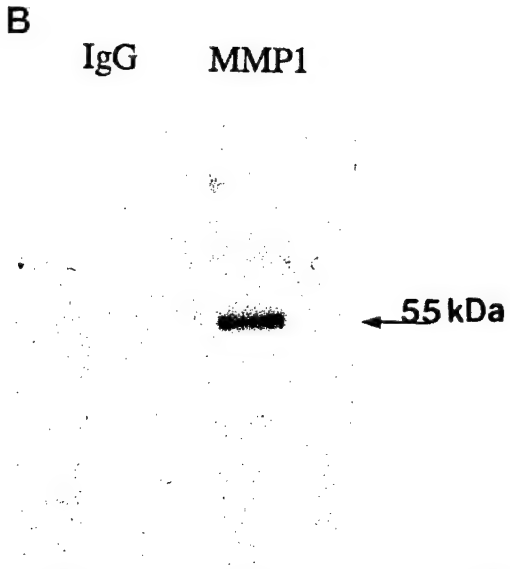


Fig. 2. EMMPRIN affinity chromatography of proteins extracted from human fibroblasts. *A*, proteins recovered from chromatography of fibroblast extracts on EMMPRIN-Sepharose were run on SDS-PAGE and silver-stained; two bands (at ~M_r 55,000 and ~M_r 67,000) were detected. *Arrows* indicate positions of the M_r 43,000 and M_r 67,000 markers. *B*, parallel gels to those in *A* were transblotted and reacted with antibody to MMP-1 or secondary antibody only (*IgG*); the ~M_r 55,000 band reacted with anti-MMP-1.

EMMPRIN preparation with antibody against MMP-1. A strong band at ~M_r 55,000, corresponding approximately in size to pro-MMP-1, reacted with the antibody, indicating the presence of MMP-1 in the EMMPRIN preparation. A weaker band at ~M_r 45,000, which is not seen consistently, is most likely activated MMP-1 (M_r 42,000).

Quantitation of the MMP-1 content by ELISA gave 2.1 μg of MMP-1 per 5 μg of total protein in the EMMPRIN preparation. Because EMMPRIN and pro-MMP-1 have molecular weights of ~58,000 and 52,000, respectively, this result suggests that EMMPRIN and MMP-1 are complexed in an equimolar ratio.

The presence of MMP-1 at the surface of LX-1 human lung carcinoma cells was confirmed by immunocytochemistry using antibody against MMP-1 (Fig. 4).

Discussion

Many recent studies have highlighted the importance of the pericellular milieu surrounding tumor cells in their proliferative and invasive behavior (6–8). This milieu is modified by a number of proteases, especially MMPs and tissue serine proteases, many of which are produced by tumor-associated stromal cells rather than tumor cells themselves (13, 14) and subsequently become concentrated at the tumor cell surface via interaction with specific binding

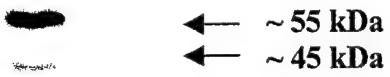


Fig. 3. Western blot of immunopurified EMMPRIN with antibody to MMP-1.

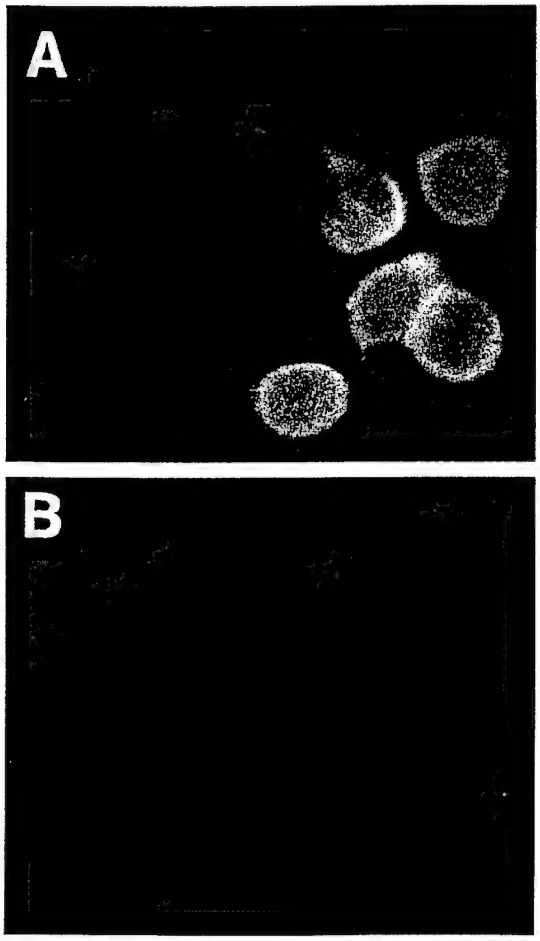


Fig. 4. Immunoreactivity of LX-1 human carcinoma cells with antibody to MMP-1. *A*, cells stained with antibody against MMP-1. *B*, cells stained with secondary antibody only.

sites. For example, MMP-2 binds to the tumor cell surface via a tissue inhibitor of MMPs-2-MT-MMP complex (15, 16). MMP-2 is activated by the MT-MMP, and the complex is targeted to invasive domains of the tumor cell membrane (sometimes termed "invadopodia") via specific docking of MT-MMP at these sites (17). Although MT-MMPs activate soluble MMP-2 as well as plasma membrane-retained MMP-2, membrane-bound enzyme is required for tumor cell invasion (17). A similar mechanism of activation and retention at the cell surface has been described for collagenase 3 (18). Other cell surface binding sites have been described for gelatinase B, *i.e.*, CD44 (19) and the α_2(IV) chain of collagen (20), and for MMP-2, *i.e.*, $\alpha_V\beta_3$ integrin (21). These sites also appear to be important in tumor cell invasion.

Evidence for association of MMP-1 with the surface of a human pancreatic carcinoma cell line has been published previously (22), but the mechanism whereby MMP-1 binds to these cells has not been described. In the present study, we show that MMP-1 binds to EMMPRIN, a tumor cell surface glycoprotein previously shown to induce synthesis of MMP-1 and other MMPs by fibroblasts (9–11) and endothelial cells.[4] We have also shown that an EMMPRIN-MMP-1 complex can be isolated from LX-1 human lung carcinoma cell membranes and that MMP-1 is present on the LX-1 cell surface. A preliminary report has been published suggesting that EMMPRIN becomes localized to invadopodia in human breast carcinoma cells (23). Tumor cell surface EMMPRIN may then be responsible for targeting MMP-1 to invadopodia, thus adding MMP-1 to the impressive list of proteases associated with these invasive structures (6, 17). Although other proteases have been shown to be important in tumor growth and invasion under a variety of conditions, it is likely that MMP-1 is crucial for penetration of fibrous tissues because of its ability to degrade fibrillar collagen as shown, for example, in endothelial cell invasion (24) and tumor cell invasion (25) of collagen gels. Thus localization of MMP-1 on the tumor cell surface via interaction with EMMPRIN would facilitate these invasive processes.

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[4] Unpublished observations.

APPENDIX 5:

Li., R., Huang, L., Guo, H., and Toole, B.P.: Basigin (murine EMMPRIN) stimulates matrix metalloproteinase production by fibroblasts. J. Cell. Physiol.**186**: 371-379, 2001.

JOURNAL OF CELLULAR PHYSIOLOGY 186:371–379 (2001)

Basigin (Murine EMMPRIN) Stimulates Matrix Metalloproteinase Production by Fibroblasts

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Analysis of basigin-null mice has shown that basigin is involved in several important physiological processes including reproductive, immune, and neural activities (Igakura et al., 1998, Dev Biol 194:152–165). However, its molecular mechanism of action in these processes has not yet been established. Our objective here is to determine whether basigin has functional properties similar to its apparent human tumor cell homolog, EMMPRIN, i.e., the ability to stimulate matrix metalloproteinase (MMP) production in fibroblasts (Guo et al. 1997, J Biol Chem 272:24–27). Mouse cells express two major forms of basigin that differ in their degree of glycosylation (molecular weights: 45 and 58 kDa) but, in similar fashion to human EMMPRIN, mouse tumor cells express higher levels of basigin than normal cells. We have used three different methods to show that basigin stimulates MMP expression in fibroblasts. First, recombinant basigin was partially purified from transfected CHO cells by affinity chromatography. This basigin preparation stimulates production of MMPs on addition to fibroblasts in culture. Second, co-culture of basigin-transfected CHO cells with fibroblasts gives rise to increased expression of MMPs as compared to control co-cultures. Third, we employed a novel approach in which a recombinant basigin adenovirus was constructed and used to infect the target fibroblasts, so that mutual stimulation between neighboring fibroblasts would be expected to result. In this method also, basigin stimulates production of MMPs. Finally, we showed that addition of basigin or EMMPRIN antibody, respectively, to recombinant basigin or EMMPRIN adenovirus-infected cells augments stimulation of MMP synthesis, implying that cross-linking of basigin/EMMPRIN in the membrane enhances activity. We conclude that murine basigin and human EMMPRIN have similar MMP-inducing activities and are functional homologs. J. Cell. Physiol. 186:371–379, 2001.

Basigin is a highly glycosylated plasma membrane protein belonging to the immunoglobulin (Ig) superfamily. It was first cloned from normal fibroblasts (Altruda et al., 1989) and from embryonic carcinoma cells (Miyauchi et al., 1990), but its function was not known at that time. Recently, a knockout mouse has been produced and the basigin-null embryos are in most cases unable to undergo implantation. However, some embryos survive past birth but these mice are sterile and have defects in sensory, immune and memory functions (Igakura et al., 1996, 1998). These analyses of the basigin knockout indicate that basigin is an important cell surface molecule in several biological processes. However, the molecular functions of basigin in these processes remain unclear.

Apparent homologs of basigin have been independently cloned from several species. The avian homolog has been implicated in blood–brain barrier and blood–eye barrier formation because of its preferential localization at these sites (Schlosshauer and Herzog, 1990; Seulberger et al., 1992) and in neural retina development since antibody blocks retinal cell interactions in culture (Fadool and Linser, 1993a). Studies of the rat and human homologs suggest that it may play a role in the activation of leukocytes (Fossum et al., 1991; Kasinrerk et al., 1992). However, the only established molecular function has come from studies of the human

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homolog named EMMPRIN. Native and recombinant human EMMPRIN stimulate matrix metalloproteinase (MMP) production in fibroblasts (Kataoka et al., 1993; Guo et al., 1997), and this stimulation of MMPs requires glycosylation (Guo et al., 1997). Moreover, cancer cells express significantly higher levels of EMMPRIN than normal cells (Muraoka et al., 1993; Polette et al., 1997; Sameshima et al., 2000) and it has been proposed that elevated expression of EMMPRIN in tumor cells may promote tumor progression by inducing MMP production in peritumoral stromal cells (Biswas et al., 1995; Toole and Zucker, 2000). Indeed, we have observed that over-expression of EMMPRIN in benign human breast tumor cells promotes tumor growth and invasion in vivo (S. Zucker, M. Hymowitz, E. Rollo, R. Mann, C. Conner, J. Cao, H. Foda, D. Tompkins, B. Toole, submitted for publication).

The basigin sequence has 58% amino acids identical to EMMPRIN but the functionally conserved amino acid residues between human and mouse increase the similarity to 95% (Miyauchi et al., 1991; Biswas et al., 1995). However, it has not yet been reported whether basigin expression is elevated in mouse tumor cells or whether basigin stimulates the production of MMPs, i.e., whether basigin and EMMPRIN are functional homologs. Answers to these questions will throw light on whether misregulation of MMPs may be involved in the defects found in basigin-null mice and will allow us to study the functions of EMMPRIN in physiological and pathological events using mouse model systems. We report here that basigin is indeed highly expressed in mouse tumor cells and that it stimulates production of MMPs in fibroblasts. We also report a novel method for assessing the activity of basigin/EMMPRIN using recombinant adenovirus constructs.

MATERIALS AND METHODS

Materials

The *N*-glycosylation inhibitor tunicamycin was purchased from Sigma and dissolved in PBS/10% DMSO. Selection antibiotic G418, culture media, and culture supplements were purchased from GIBCO, and fetal bovine serum (FBS) from Hyclone. Polyclonal antibody #2881 was raised using polypeptide aa68–82 of basigin as antigen. Antibody against human EMMPRIN (CD147), HIM6, was obtained from Pharmingen. Anti-polyhistidine antibody was purchased from Invitrogen, and anti-hemoagglutinin (HA) antibody from Roche Biochemical. All horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Amersham-Pharmacia (Piscataway, NJ).

Cell culture

All cells were cultured at 37°C in 95% air and 5% CO_2. CHO-K1 cells, G8 and C2C12 myoblasts, 293 cells, CCD-16 and CCD-18 fibroblasts were purchased from American Type Cell Culture. The TA3/St cell line was established from an ascites subline originally derived from a spontaneous mouse mammary adenocarcinoma (Nagy et al., 1995). NHF III fibroblasts were prepared in our lab from human foreskin. CHO-K1 cells were cultured in F12/10% FBS, stable transfectant CHO cells in F12/10% FBS supplemented with 0.5 mg/ml G418, G8 myoblasts in DMEM/10% FBS/10% horse serum, and CCD-16 cells in DMEM/F12/10% FBS supplemented with non-essential amino acids and sodium pyruvate. All other cells were cultured in DMEM/10% FBS. For tunicamycin treatment, TA3 cells grown to confluence were used with or without infection with recombinant basigin adenovirus and treated with 2–8 μg/ml of tunicamycin in normal culture media for 16 h. The cells were then lysed and lysates were used for Western blotting to detect basigin (see below).

Western blots

Cells were lysed in RIPA buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxylcholate, 0.1% SDS, 1 mM EDTA, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF). Protein was quantitated using BioRad protein assay reagents. Cell lysates were subjected to SDS-PAGE with 10% or 12% gels under reducing conditions. Pre-stained molecular markers (GIBCO, Grand Island, NY) were used to estimate the molecular weight of samples. Proteins were transferred to Hybond-ECL membrane (Amersham-Pharmacia) in running buffer with 20% methanol. After non-specific sites were blocked with 5% non-fat dry milk/1% host animal serum of secondary antibody in TBST (20 mM Tris–HCl, pH 7.6, 0.5 M NaCl, 0.1% Tween-20) for 30 min at room temperature, the membrane was then incubated in TBST/5% non-fat dry milk with antibody #2881 (rabbit anti-basigin, 1:4,000), mouse Mab against polyhistidine (1:100) or rat anti-HA tag antibody (0.08 μg/ml) for 1 h at room temperature. After three washes with TBST, the membranes were incubated with appropriate HRP-conjugated secondary antibody for 1 h in TBST/5% non-fat dry milk/1% animal serum. Protein bands were visualized with chemiluminescence reagents (DuPont NEN, Boston, MA) and by exposure on Kodak X-film according to manufacturer's instructions.

Cloning of basigin

Basigin cDNA in pBluescript was cloned into eukaryotic expression vector PCR-3 (Invitrogen) by PCR using primers Bas5′ (5′-CTC CTG GAC GAG GCG ACA TG-3′) and Bas3′(5′-TCA ATG ATG GTG ATG GTG ATG GGT GGC GTT CCT CTG GCG-3′). The underlined sequence codes six histidines; this polyhistidine was placed at the C-terminal of basigin to facilitate detection and purification. The sequence of the construct was confirmed using a Sequenase kit (USB) according to manufacturer's instructions.

Transfection and selection of stable clones

All transfections were done using GIBCO's TransfectaminePlus kit according to manufacturer's instructions. After 5 h transfection, cells were then cultured in regular culture media (with FBS). After 24 h, the transfected cells were washed, trypsinized and plated into 100 mm culture dishes and selected for stable clones using regular media supplemented with 500 μg/ml G418. After 2–4 weeks, clones were picked using cloning cylinders (Sigma). Clones were amplified by step-wise culturing of the cells in 12-well plates, 6-well plates, and 100 mm dishes. The expression of basigin in these clones was measured by Western blotting.

Purification of recombinant basigin

Recombinant basigin tagged with polyhistidine was purified using chromatography on a nickel-affinity column. Twenty to forty 100 mm dishes of stable transfectant BAS4-CHO cells were grown to confluence, lysed in RIPA buffer and the supernatant from the lysate was collected. Imidazole solution was added to the supernatant to reach a final concentration of 10 mM. Recombinant basigin was then purified from this solution using a Hitrap column (nickel charged, Pharmacia) according to the manufacturer's instructions. A mock control preparation was made in the same fashion from vector transfectants. The presence of basigin in collected fractions was measured by Western blotting. The purity of basigin was assessed by SDS-PAGE and silver staining using a kit (Diagnostic) according to manufacturer's instructions.

Preparation of recombinant adenoviruses

Basigin cDNA was amplified by PCR with primers Bas5′ (5′ ACT GAA TTC ACG AGG CGA CAT GGC GGC GCT 3′) and BasTag (5′ ACT TCT AGA TCA ATG ATG GTG ATG GTG ATG ATG AGC ATA ATC TGG AAC ATC ATA TGG ATA GGT GGC GTT CCT CTG GCG TAC 3′). EMMPRIN cDNA was amplified using primers EMMP5′ (5′ GAC GAA TTC GAG GAA TAG GAA TCA TGG CGC CT 3′) and EMMPTag (5′ ACT TCT AGA TCA ATG ATG GTG ATG GTG ATG AGC ATA ATC TGG AAC ATC ATA TGG ATA GGA AGA GTT CCT CTG GCG GAC 3′). Th BasTag and EMMPTag primers contain sequences (underlined) that code hemoagglutinin (HA) and polyhistidine (His) tags. The amplified cDNAs were then purified and cloned into the shuttle vector PACCMV-pLpA at the *ECORI* and *Xba*I restriction sites. The adenovirus genome plasmid PJM-17 was transformed into 5H-α. Clones were picked and inoculated into 3 ml of LB bacteria media. The small culture was transferred into a large culture with 100 μg/ml ampecillin and 0.5% Glucose. PJM-17 was then purified from this culture using Qiagen's midi or maxi plasmid purification kit.

For co-transformation, 293 cells were grown to confluence in 60 mm dishes. The cells then were transformed with 5 μg of PJM-17 and 5 μg of PACCMV-pLpA-basigin plasmid using Pharmarcia's CellPhect transfection kit. After 6 h of transfection, the cells were washed three times with PBS and cultured with normal 293 media. The media were changed every 6 days. Formation of the recombinant viruses and cell lysis occurred after 2–4 weeks. The lysed cell and media were combined and centrifuged, and the supernatant was collected for cloning and titration.

To clone the viruses, 293 cells were grown to confluence in 60 mm dishes and infected with 1 ml of media-diluted (10^{-6}–10^{-9}) virus preparation for 1 h at 37°C. The cells were then layered with 0.6% agar prepared by mixing 1.2% agar with 2× media right before use. The dishes were placed at room temperature for 15 min and then at 37°C for 3–5 days, at which time plaques formed and were counted to calculate the original virus titer. For clone picking, a sterile glass pipet was used to suck up the agar in a single plaque area. The cloned viruses were released by three cycles of freeze-thawing. To amplify the viruses, 293 cells were grown to confluence and infected with recombinant virus at a cell:virus ratio of about 1:10. After 24–48 h, when 10–20% of cells were lysed, the 293 cells were washed twice with PBS and scraped into 3–10 ml of PBS/10% glycerol. The cells were lysed by six cycles of freeze-thawing and the lysate was then centrifuged at high speed. The supernatant was collected, titrated, and stored at −80°C. The viruses are termed Adv-Bas and Adv-EMMP, respectively. A control virus expressing β-galactosidase (Adv-Gal) was obtained from Dr. Kenneth Walsh, Tufts University (Perlman et al., 1998).

Measurement of MMP production

Fibroblasts (NHF III or CCD-16) were cultured in 24-well plates to confluence. The fibroblasts were then washed with serum-free medium. Purified basigin or mock purification control was added into the culture in DMEM/2% FBS. After 2 days, the media were collected and subjected to ELISA using collagenase (MMP-1) and stromelysin (MMP-3) BioTrak kits (Amersham-Pharmacia) according to manufacturer's instructions.

For co-culture experiments, CHO cells stably transfected with basigin or vector alone were cultured with fibroblasts at a ratio of 2:1 (CHO:fibroblast) for 24 h in DMEM/F12/10% FBS. The media were then changed to DMEM/F12/2% FBS. After another 48 h, media were collected and duplicate samples measured by ELISA.

When using recombinant viruses, CCD-16 cells grown to confluence were infected with recombinant basigin adenovirus (Adv-BAS), EMMPRIN adenovirus (Adv-EMMP), or control β-galactosidase adenovirus (Adv-Gal) for 4 h, then media were changed to DMEM/2% FBS with or without other reagents. After 3 days, media were collected and duplicate samples measured by ELISA.

RESULTS

Highly glycosylated basigin is expressed at high levels in mouse tumor cells

Based on a hydrophobicity plot of the amino acid sequence of basigin, a peptide region (aa68–82) located in the first Ig domain was chosen to raise a polyclonal antibody (#2881) against basigin. We verified that antibody #2881 recognizes basigin and is specific for basigin by performing Western blots of lysates obtained from fibroblasts infected with recombinant basigin adenovirus in which the basigin is tagged with HA. As can be seen in Figure 1, antibody #2881 reacted with ~58 and ~45 kDa species of protein. Antibody against the HA tag gave a virtually identical pattern of staining indicating that antibody #2881 recognized basigin. This conclusion is confirmed by the complete lack of staining by either antibody in lysates of fibroblasts infected with recombinant β-galactosidase adenovirus. Thus, antibody #2881 was used to measure the expression of basigin in the following experiments.

Western blotting showed that basigin is expressed in all of the different mouse cell lines measured, but at different levels. TA3/St breast carcinoma cells express the highest levels and B16 melanoma cells also express relatively large amounts (Fig. 2). However, G8 myoblasts, C2C12 myoblasts and 3T3 cells express much

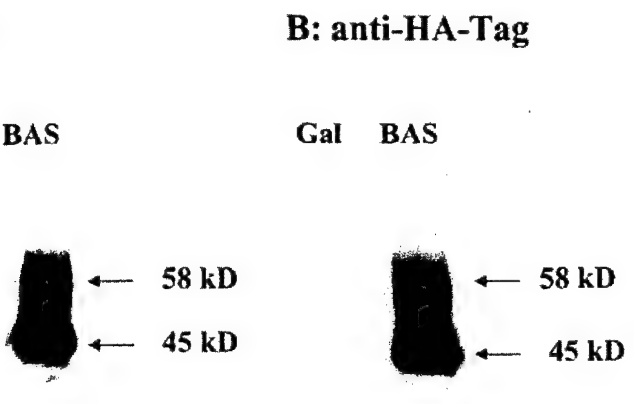

Fig. 1. Antibody #2881 recognizes basigin. NHF-III fibroblasts were infected with recombinant basigin (BAS) or control β-galactosidase (Gal) adenovirus. Western blots were performed with cell lysates, using: (**A**) rabbit polyclonal antibody #2881 against basigin-derived peptide; (**B**) rat monoclonal antibody against HA-tag. Both antibodies recognized polydisperse proteins of molecular weights ~58 and ~45 kDa from the Adv-BAS-infected cells. In neither case was a signal obtained with Adv-Gal-infected cells. Similar results were obtained with Adv-BAS-infected CCD-16 cells (Fig. 8) and with basigin-transfected CHO cells (Fig. 4).

lower amounts of basigin than the tumor cells (Fig. 2). All mouse cell lines measured express the 45 kDa band reported before (Altruda et al., 1989), but they also express a 58 kDa form of basigin, which is a similar molecular weight to the active MMP-stimulatory form of human EMMPRIN. We have observed that the relative amounts of 45 and 58 kDa forms vary (e.g., compare Fig. 2, lane 2 and Fig. 3A, lane 1) but we have not been able to associate this variability with culture conditions such as cell density, number of passages, etc. However, the tumor cells always make more of the 58 kDa than the 45 kDa form.

Previous results (Miyauchi et al., 1990; Fadool and Linser, 1993b; Guo et al., 1997) have shown that basigin and its homologs are highly glycosylated. To confirm that the 45 and 58 kDa species are different glycosylation forms of the same protein, TA3/st cells were treated with tunicamycin for 16 h to specifically inhibit *N*-glycosylation. This experiment was performed with wild type TA3 cells and cells infected with recombinant basigin adenovirus. In both cases, a single new band of molecular weight similar to that predicted for the non-glycosylated polypeptide was visualized by Western blotting after tunicamycin treatment, concomitant with loss of protein from the two original bands (Fig. 3). However, greater conversion of glycosylated to non-glycosylated form was observed for the adenovirus-infected cells. This is presumably due to the fact that only newly synthesized, epitope-tagged basigin is detected in the virus-infected cells. These results confirm that both forms of basigin are derived from a single polypeptide.

Recombinant basigin produced in mammalian cells is similar to native basigin

Purified native human EMMPRIN from LX-1 lung carcinoma cells stimulates MMP production by fibroblasts (Kataoka et al., 1993). Due to lack of glycosylation, recombinant human EMMPRIN produced from bacteria has a much lower molecular weight than EMMPRIN purified from human LX-1 tumor cells and has no activity. Also, intermediate size products expressed in COS cells appear to be inactive (Guo et al., 1997). However, fully glycosylated human recombinant protein of about 58 kDa expressed in transfected CHO cells has activity similar to LX-1 derived native protein.

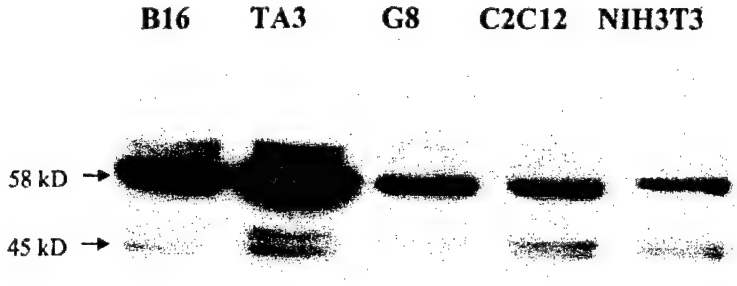

Fig. 2. Mouse tumor cells express higher levels of basigin than normal cells. B16 melanoma cells, TA3/St breast carcinoma cells, G8 myoblasts, C2C12 myoblasts and NIH-3T3 cells were grown to confluence then lysed in RIPA buffer. Cell lysates were analyzed by Western blotting with anti-basigin antibody #2881. Fifty micrograms of total protein was loaded for each lysate.

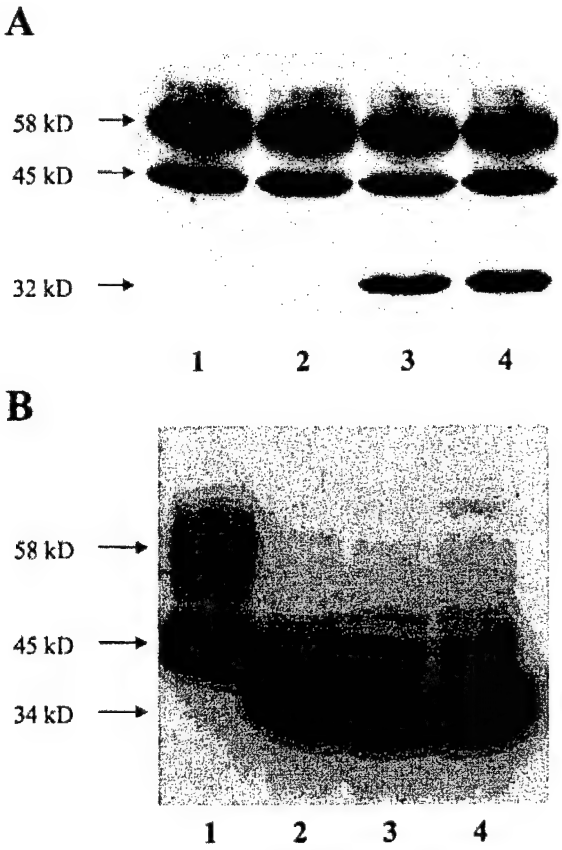

Fig. 3. Both molecular weight forms of basigin are glycosylated. **A**: TA3/St cells grown to confluence were treated with or without tunicamycin for 16 h. Cells were then lysed in RIPA buffer and used for Western blotting with antibody #2881. A single new band of ~32 kDa appeared after tunicamycin treatment. Lane 1: no treatment. Lane 2: 0.04% DMSO. Lane 3: 2 μg/ml tunicamycin (in 0.04% DMSO). Lane 4: 4μg/ml tunicamycin. **B**: TA3/St cells were infected with Adv-BAS (tagged with HA) for 4 h and then cultured with or without tunicamycin for 16 h. Recombinant basigin was then measured by Western blotting with anti-HA antibody. A single new band of ~32 kDa appeared. Lane 1: no treatment. Lane 2: 2 μg/ml tunicamycin. Lane 3: 4 μg/ml tunicamycin. Lane 4: 8 μg/ml tunicamycin.

It has therefore been concluded that post-translational modification is required for EMMPRIN activity (Guo et al., 1997). Based on these studies of human EMMPRIN, we decided to characterize the function of basigin by expressing recombinant basigin in mammalian cells. Two primers, BAS5′ and BAS3′, were synthesized to amplify basigin cDNA, the 3′ primer containing sequences encoding six histidines. This polyhistidine tag was placed at the C-terminus of basigin to facilitate purification by nickel-column chromatography. Amplified basigin cDNA was then cloned into the eukaryotic expression vector PCR-3, which contains a selection marker (neomycin resistance). Transfection of this construct into COS cells produced incompletely glycosylated basigin, but transfection of the construct into CHO cells gave rise to 42 and 58 kDa forms (Fig. 4), similar in size to that found above. However, the 42 kDa form is slightly smaller than the 45 kDa form detected in mouse tumor cells (Fig. 2). As noted above, this kind of cell type-specific post-translational modification was also observed for human EMMPRIN. CHO stable-transfectant clones were obtained by G418 selection. One of these clones, BAS4-CHO, which gave the highest level of basigin expression, was used for subsequent purification of basigin and for co-culture experiments.

Recombinant basigin stimulates the production of MMPs by fibroblasts

Recombinant basigin was partially purified from a large scale culture of BAS4-CHO cells. Several lysis, loading, and elution conditions were tried to develop optimal conditions for affinity chromatography on a nickel-charged column, using antibody #2881 to monitor the purification process. In this way basigin of relatively high purity was obtained from transfectant CHO cell lysates. However, the yield was low and at least two additional bands were detected in the final basigin preparation (Fig. 5). This partially purified basigin was concentrated and the detergent was removed by repeated ultrafiltration. We then tested its effect on MMP-1 and MMP-3 production by human fibroblasts known to respond to human EMMPRIN, i.e., CCD-16 and NHF III cells (Guo et al., 1997). CCD-16 cells produce very little background MMPs but stimulation of MMP production by basigin was modest. Early passages of NHF III cells respond better to basigin although they display higher background expression of MMP-1 and MMP-3. Stimulation of MMP-1 in NHF III cells by 0.8 μg/ml basigin was ~5-fold, i.e., ~50% of maximal stimulation by the phorbol ester, phorbol 12-myristate 13-acetate (Frisch et al., 1987); ~2-fold stimulation of MMP-3 was also obtained (Fig. 6).

Co-culture experiments using CHO transfectant cells and NHF III fibroblasts were performed to confirm the

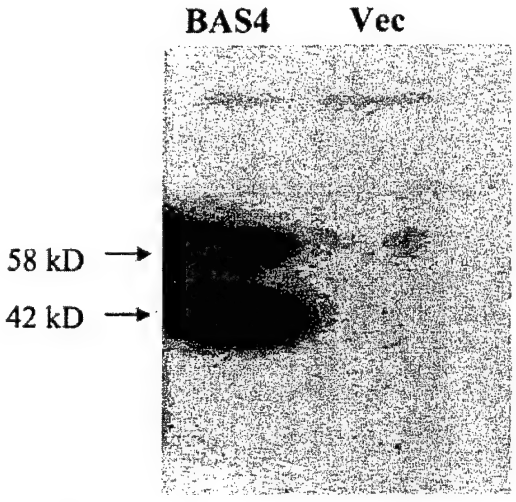

Fig. 4. Stable expression of recombinant basigin in CHO cells. PCR-3-basigin was constructed by standard methods and transfected into CHO cells. Lysates from basigin and vector stable transfectant clones were analyzed by Western blotting with antibody #2881. Several clones were analyzed and shown to express basigin. One of the clones (BAS4) is shown here compared with one of the stable transfectants with vector only (Vec). Two forms of recombinant basigin were expressed with molecular weights, ~42 and ~58 kDa in the former but not the latter.

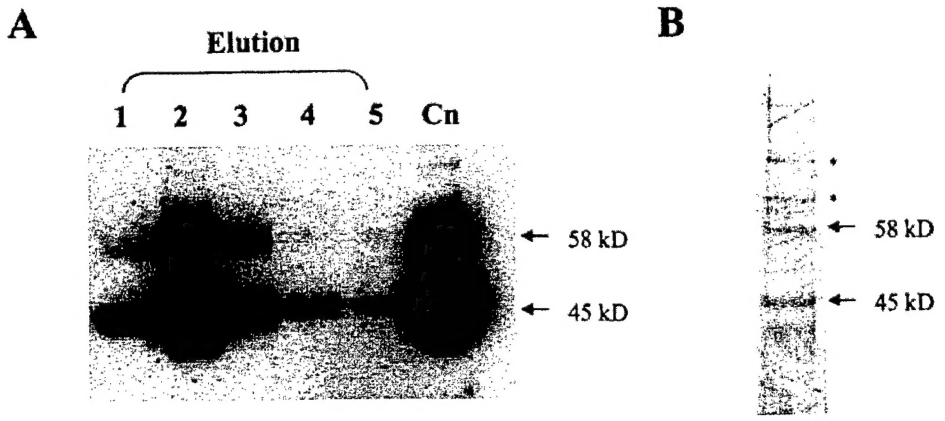

Fig. 5. Purification of basigin from BAS4-CHO cells by nickel affinity chromatography. BAS4-CHO cells were lysed in RIPA buffer with 10 mM imidazole and basigin was purified from the lysate on a nickel-charged affinity column. **A**: Western blot with antibody #2881 of eluted fractions (1–5) and pooled fractions 2 and 3 after concentration for SDS-PAGE/silver staining and for measurement of activity (Cn). **B**: Silver staining of the partially purified basigin (Cn). Arrows indicate the 58 and 45 kDa forms of basigin. Asterisks indicate two additional, unknown protein bands.

result obtained with partially purified basigin. Different ratios of the two cell types were used and a ratio of transfectant CHO cells to fibroblasts of 2:1 was found to be optimal. Under these conditions, co-culture of BAS4-CHO and NHF III cells stimulates the production of MMP-1 and MMP-3 by 2- to 3-fold, as compared to co-culture of CHO cells transfected with vector only and NHF III cells (Fig. 7).

Infection with recombinant basigin adenovirus induces MMP production

Recombinant basigin adenovirus (Adv-BAS) was used to determine whether infection of fibroblasts would cause a high level of basigin expression that would in turn induce an increase in MMP production. The principle behind this approach is that basigin expressed by neighboring fibroblasts should bind *in trans* to the putative basigin receptor and stimulate each other to produce MMPs. Infection with Adv-BAS was shown to cause a high level of expression of basigin of 45 and 58 kDa in NHF III (Fig. 1) and CCD-16 fibroblasts (Fig. 8A) and, as expected, MMP production was stimulated in both cell types. However, CCD-16 cells were used for most of these experiments since they were easier to infect with adenoviruses and since they produce very low background levels of MMPs. Infection of the fibroblasts with Adv-BAS induces a high level of production of MMP, while infection with control virus (Adv-Gal) has no effect on MMP production (Fig. 8B). The stimulation obtained was even more convincing (>10-fold for MMP-3) than that obtained in co-culture with basigin-transfected CHO cells (2–3-fold). This result further confirms the MMP-stimulating activity of basigin.

Antibodies to basigin and EMMPRIN augment MMP stimulation

We found that antibody #2881, but not control rabbit IgG, enhances the stimulation of MMP by Adv-BAS. This enhanced MMP stimulation is abolished by the peptide used to raise #2881, indicating that this is a specific response (Fig. 9A).

To ensure that the above phenomenon was not restricted to the murine homolog, basigin, we also constructed a recombinant adenovirus for human EMMPRIN. When the CCD-16 cells were infected with

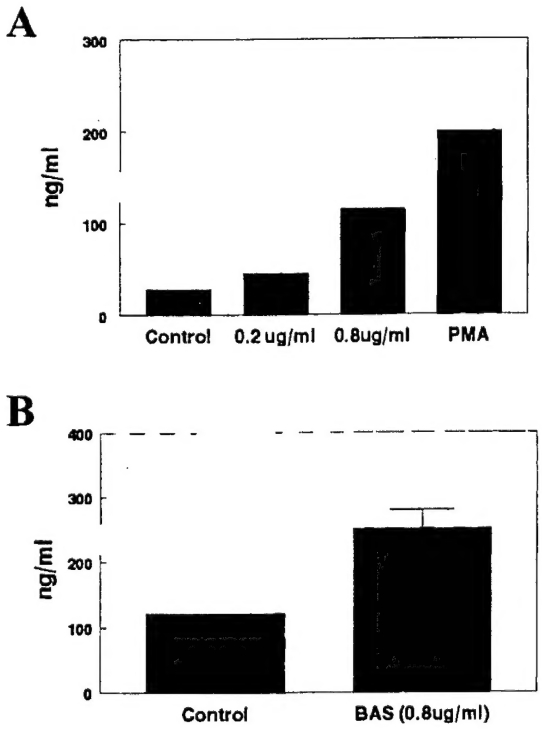

Fig. 6. Recombinant basigin stimulates production of MMP-1 and MMP-3 by fibroblasts. NHF III fibroblasts grown to confluence were treated with partially purified basigin or a mock purification preparation for 48 h in DMEM/2% FBS. Conditioned media were collected for measurement of MMP-1 and MMP-3 production by ELISA. **A**: MMP-1; due to the small amount of material available, only single aliquots of 0.2 and 0.8 μg/ml of basigin were used in this analysis. **B**: MMP-3 (data are means ± range of duplicate measurements). Phorbol 12-myristate 13-acetate (PMA) was used as a positive control (Frisch et al., 1987).

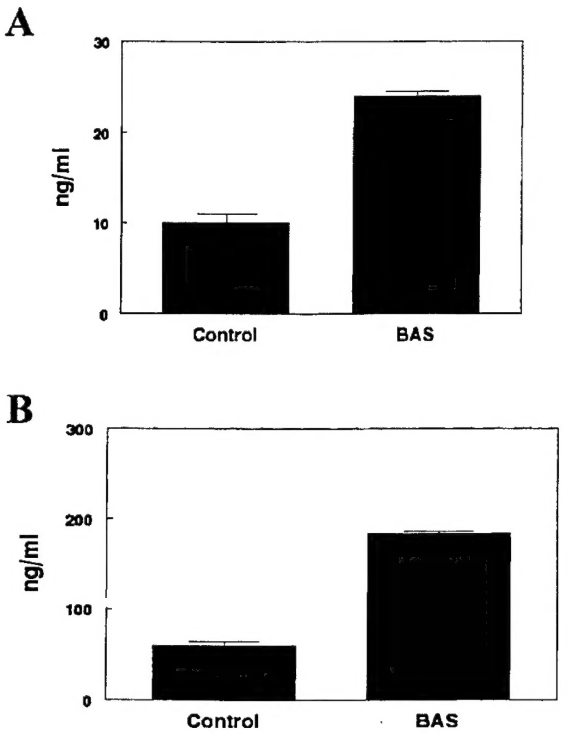

Fig. 7. Co-culture of fibroblasts with basigin-transfected (BAS4) CHO cells increases the production of MMPs. NHF III fibroblasts were plated together with BAS4-CHO cells or control vector-transfected CHO cells at a 2:1 ratio (CHO: fibroblasts) for 24 h in DMEM/F12/10% FBS. Media were then changed to DMEM/2% FBS. After 48 h, media were collected for ELISA. **A**: MMP-1. **B**: MMP-3. In A and B, data are means ± range of duplicate measurements.

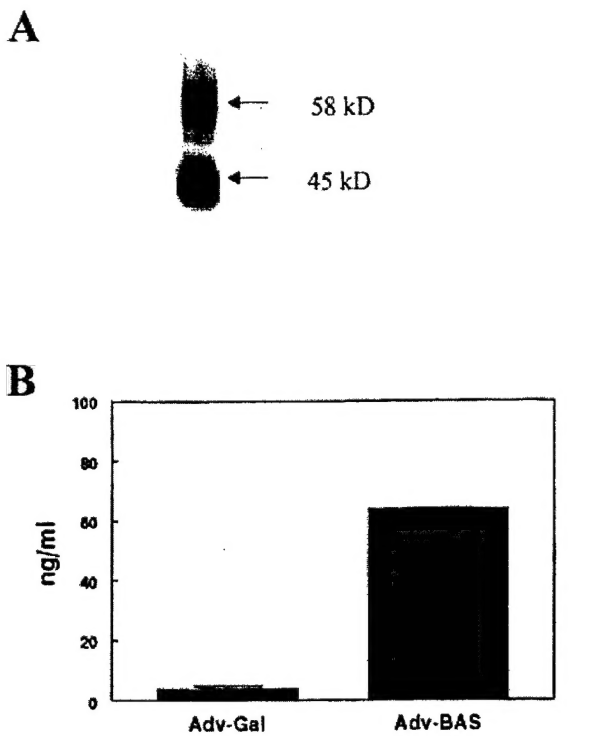

Fig. 8. Infection of fibroblasts with recombinant basigin adenovirus (Adv-BAS) stimulates the production of MMP. **A**: Infection of Adv-BAS into CCD-16 cells results in a high level of expression of basigin. CCD-16 cells grown to confluence were infected with Adv-Bas for 24 h and lysed in RIPA buffer. Basigin expression was measured by Western blotting with anti-HA tag antibody. Basigin of molecular weight ~58 and ~45 kDa was detected; no signal was obtained on infection with control recombinant β-galactosidase adenovirus (Adv-Gal) (not shown). **B**: Infection with Adv-BAS stimulates MMP production. CCD-16 cells grown to confluence were infected with Adv-BAS or Adv-Gal for 4 h. Media were then changed to DMEM/2% FBS. After 3 days, media were collected for assay with the MMP-3 ELISA kit (data are means ± range of duplicate measurements). Similar results to those shown here were obtained with MMP-1.

this adenovirus, MMP production was stimulated in a fashion similar to that obtained with Adv-BAS, and this stimulation was also augmented by the addition of monoclonal antibody against EMMPRIN (Fig. 9B).

DISCUSSION

In this study, we have shown that murine basigin has similar functional properties to human EMMPRIN, establishing that they are functional homologs. As previously found for EMMPRIN (Guo et al., 1997; Polette et al., 1997; Sameshima et al., 2000), basigin expression is elevated in tumor cells and basigin stimulates production of MMP-1 and MMP-3 in fibroblasts. During early investigations of tumor–stromal cell interactions, it was found that mouse B16 melanoma cells stimulate MMP production by fibroblasts (Biswas, 1982) and that this activity is located on the plasma membrane of B16 cells (Biswas and Nugent, 1987). Here we have shown that B16 cells, as well as TA3/St mammary carcinoma cells, express high levels of basigin, which is presumably the cell surface MMP-stimulating activity that was originally observed and that led to molecular characterization of human EMMPRIN (Biswas et al., 1995). We have also shown that antibody to basigin or EMMPRIN greatly enhances their ability to stimulate MMP synthesis. Thus, it is likely that cross-linking of basigin within the cell membrane enhances its activity. Past studies have shown that basigin and its chicken homolog form homo-oligomers in the plasma membrane (Fadool & Linser, 1996; Yoshida et al., 2000). These results suggest the possibility that physiological polymerization within the membrane enhances EMMPRIN activity.

Analyses of basigin-null ($Bsg^{-/-}$) mice have revealed numerous physiological defects (Igakura et al., 1996, 1998). Thus, a question of significant interest is whether alteration in regulation of MMP synthesis during these processes could explain these defects or whether basigin/EMMPRIN acts via additional molecular mechanisms. One of the defects in $Bsg^{-/-}$ mice is that few embryos successfully implant and consequently most $Bsg^{-/-}$ embryos die at the peri-implantation stage. Basigin is strongly expressed in trophoectoderm of wild-type mouse embryos and the wild-type uterus at days 3.5–7.5 of gestation (Igakura et al., 1998). Thus, the phenotype of $Bsg^{-/-}$ embryos is consistent with the pattern of basigin expression around the time of implantation, which occurs at approximately Day 4.5. Furthermore, transfer of normal blastocysts into the uteri of $Bsg^{-/-}$ mice results in the loss of most embryos

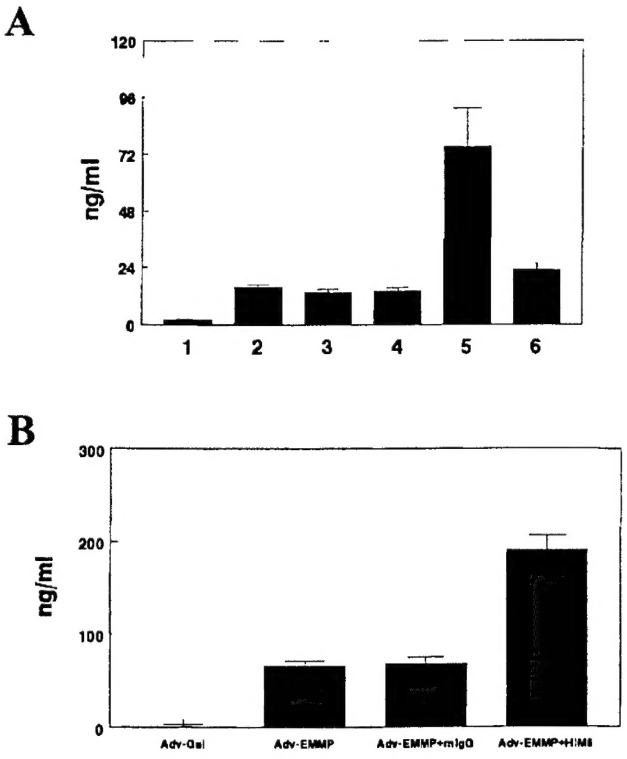

Fig. 9. Antibody against basigin/EMMPRIN enhances recombinant basigin/EMMPRIN adenovirus-stimulated MMP production. CCD-16 cells grown to confluence were infected with recombinant adenoviruses for 4 h. The media were then changed to DMEM/2% FBS with or without specified reagents. After 3 days, media were collected for assay with the MMP-3 ELISA kit. **A**: Basigin. Polyclonal antibody #2881 against mouse basigin (50 μg/ml) but not rabbit IgG enhances Adv-Bas-stimulated MMP-3 production. This enhancement can be abolished by 100 μg/ml of the peptide used to raise #2881. 1: Adv-Gal. 2: Adv-BAS. 3: Adv-BAS + peptide. 4: Adv-BAS + rabbit IgG. 5: Adv-BAS + #2881. 6: Adv-BAS + #2881 + peptide. **B**: EMMPRIN. Monoclonal antibody HIM6 against human EMMPRIN (2 μg/ml) but not mouse IgG enhances Adv-EMMP-stimulated MMP-3 production. All data are means ± range of duplicate measurements.

during early pregnancy (Kuno et al., 1998). Given that migration of blastocysts is MMP-dependent and that embryo invasion into the uterine epithelium and stroma is at least partially dependent on MMPs (Brenner et al., 1989; Alexander et al., 1996), failed implantation of $Bsg^{-/-}$ embryos may be due to disturbed MMP regulation. From the above observations it is reasonable to postulate that basigin regulates the production of MMPs during embryo implantation and that disturbed MMP expression leads to defective implantation in $Bsg^{-/-}$ mice. However, evaluation of MMP expression in $Bsg^{-/-}$ uteri has not yet been performed.

Although it is likely that stimulation of MMP production underlies the function of basigin in implantation, the other defects observed in $Bsg^{-/-}$ embryos are not as readily explained in this way and suggest that basigin may have more than one function. For example, male $Bsg^{-/-}$ mice are sterile due to the lack of mature sperm. This is in agreement with the observation that basigin mRNA is strongly expressed in spermatocytes and spermatids of wild-type mice (Igakura et al., 1998) and the previous finding that CE9, the rat homolog of basigin, is synthesized throughout but dynamically redistributed during spermatogenesis (Nehme et al., 1993). $Bsg^{-/-}$ mice also show lowered sensitivity to irritating odors (Igakura et al., 1996) and deficits in spatial learning and memory (Naruhashi et al., 1997), indicating that basigin may have significant functions in development of the nervous system. In keeping with this possibility, it has been shown that the chicken homolog of basigin is expressed in immature neurons but not mature neurons (Schlosshauer, 1991) and is specifically localized at the site of the blood–brain barrier and blood–eye barrier (Schlosshauer and Herzog, 1990; Seulberger et al., 1992). This homolog also influences differentiation of Muller glial cells, whose maturation is dependent on neuron–glial contact (Fadool and Linser, 1993a). These observations imply that basigin/EMMPRIN is involved in structural or signaling aspects of cell–cell interactions. However, it is also conceivable that MMPs, produced under the influence of basigin/EMMPRIN, bring about proteolytic processing of regulatory factors necessary for these events (Werb, 1997; Peschon et al., 1998). It will be of great interest to determine, in future studies, whether the numerous biological functions discussed above can be explained by the ability of basigin/EMMPRIN to induce MMP production or whether it has multiple molecular actions.

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